INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.

University Microfilms International

300 N. ZEEB ROAD, ANN ARBOR, MI 48106
18 BEDFORD ROW, LONDON WC1R 4EJ, ENGLAND

8006446

HUANG, CHONG JEN

THE ROLE OF CYCLIC AMP MEDIATED DEPHOSPHORYLATION AND CYCLIC AMP MEDIATED PHOSPHORYLATION IN THE MECHANISM OF ACTION OF ANTI-DIURETIC HORMONE

City University of New York PH.D. 1979

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106 18 Bedford Row, London WC1R 4EJ, England

Copyright 1979

by

Huang, Chong Jen

All Rights Reserved

©

COPYRIGHT BY

CHONG-JEN HUANG

1979

THE ROLE OF CYCLIC AMP MEDIATED DEPHOSPHORYLATION AND CYCLIC AMP MEDIATED PHOSPHORYLATION IN THE MECHANISM OF ACTION OF ANTI-DIURETIC HORMONE

by

CHONG-JEN HUANG

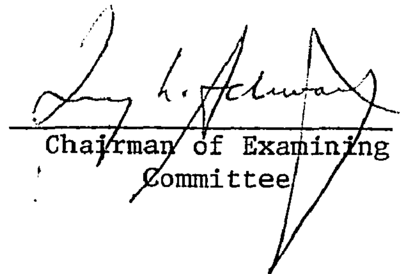
Advisor: Professor I.L. Schwartz

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

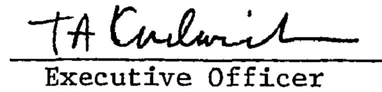
1979

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

8/28/79
Date


Chairman of Examining Committee

8/31/79
Date


Executive Officer

The City University of New York

To my Father and Mother

ABSTRACT

THE ROLE OF CYCLIC AMP MEDIATED DEPHOSPHORYLATION
AND CYCLIC AMP MEDIATED PHOSPHORYLATION
IN THE MECHANISM OF ACTION OF ANTI-DIURETIC HORMONE

by

Chong-Jen Huang

Advisor: Irving L. Schwartz, M.D.

The studies reported in this dissertation were directed at the evaluation of the role of cyclic AMP-dependent protein kinase, protein phosphatase and phosphorylated plasma membrane protein in the action of antidiuretic hormone (ADH) and the elucidation of the mechanism of cyclic AMP-dependent dephosphorylation in renal tissue.

Protein was solubilized from papillary plasma membrane (PPM), and two membrane-bound protein kinases were separated by chromatography. The major peak was identified as MPK II (M_r = 110,000) and the minor peak, as MPK I (M_r = 210,000). Both membrane-bound protein kinases are cyclic AMP-dependent and bind cyclic AMP, and the activities of both kinases are inhibited by heat-stable inhibitor, but only MPK II manifests autophosphorylation.

Two cyclic AMP-dependent protein kinases were separated from the cytosol fraction of the renal medulla by chromatography. The first peak, the minor one, was identified as CPK I, and the second peak, the major one, as CPK II (M_r = 180,000). Both cytosol protein kinases are cyclic AMP-dependent and bind cyclic AMP, but only CPK II manifests autophosphorylation. Corresponding cytosol protein kinases with similar characteristics were found in the renal cortex.

The membrane-bound phosphatase activity was separated into two peaks by chromatography. The major membrane-bound protein phosphatase was identified as Ma (M_r = 33,000) and the minor one, as Mb (M_r = 108,000). Two protein phosphatases were separated from the cytosol fraction of the renal medulla by chromatography. The major peak was identified as Pa (M_r = 35,000) and the minor one, as Pb (M_r = 100,000). The phosphatase activity is stimulated by Mn^{++} and inhibited by ATP. The ethanol treatment of Pa did not result in any change in phosphatase activity, whereas ethanol treatment of Pb resulted in a 90% increase in activity. When compared to the cytosol phosphatases, Pa and Pb, the membrane-bound phosphatases, Ma and Mb, have similar chromatographic characteristics and manifest similar behavior after treatment with ethanol. Chromatography of cytosol protein phosphatase from renal cortex resolved two peaks of activity similar to those separated from the cytosol fraction of renal medulla. In the cytosol fraction from renal cortex, however, there was a Mn^{++}-dependent protein phosphatase that was activated only in the presence of Mn^{++}.

Incubating isolated papillary plasma membrane (PPM) with ATP resulted in endogeneous phosphorylation, which was increased by cyclic AMP. Purified protein kinases, MPK II and CPK II, or the catalytic subunit of these kinases were capable of phosphorylating PPM, and isolated renal protein phosphatase dephosphorylated PPM. PPM-bound proteins with molecular weights of 38,000, 50,000 and 100,000 are phosphorylated, and cyclic AMP slightly enhances the phosphorylation of these proteins. The major protein that is phosphorylated is

characterized by a molecular weight of 50,000 dalton and is designated protein P-50. The characteristics of P-50 can be summarized as follows: 1) In the absence of cyclic AMP, it is phosphorylated. This phosphorylation is not affected by the presence of phosphatase. 2) In the presence of cyclic AMP the phosphorylation of P-50 is enhanced provided that phosphatase activity is negligible in the assay system. On the other hand, P-50 is dephosphorylated in the presence of cyclic AMP when phosphatase activity is manifest in the system. Purified MPK II and CPK II both manifest autophosphorylation and cyclic AMP-dependent dephosphorylation, and the autophosphorylated moieties of both of these enzymes have a molecular weight of 50,000 and can be identified as the regulatory subunit of protein kinase.

As in the case of cyclic AMP-dependent protein kinase from cardiac muscle, the phosphorylated renal holoenzyme (RC) appears to be a poorer substrate for renal protein phosphatase than its dissociated phosphorylated regulatory subunit (R). Thus, the cyclic AMP-induced dissociation of R from RC renders the phosphorylated R moiety susceptible to protein phosphatase action, thereby explaining the cyclic AMP-dependent dephosphorylation of protein P-50.

ACKNOWLEDGMENT

I would like to express my profound gratitude to my advisor, Dr. Irving L. Schwartz, whose gracious guidance, encouragement and support throughout by career as a graduate student made this work possible. I thank him for his trust and confidence in me, for the help and advice he has given me during the course of my research, for aid in the preparation of this dissertation, for the many opportunities he provided to me to meet distinguished scientists and, above all, for the privilege of working with him.

I would also like to address my very special thanks to Dr. Herman R. Wyssbrod, who has constantly offered valuable advice, supported my work and helped me solve personal problems, to Dr. Heng-Chun Li, who trained me in basic biochemical research techniques and advised me in many of the biochemical aspects of this work, to Dr. Pierre Ripoche, with whom I worked during his sabbatical year at Mount Sinai on a day-to-day basis and from whom I received valuable advice and training in many basic physiological research techniques, to Dr. William A. Brodsky for his cogent discussions and cooperation, and for the example he provided of true love for basic research. I would also like to express my gratitude to the following: (1) Eric Scalattar, Lonny Reisman and Lisa Roth for their excellent assistance and collaboration, (2) Drs. Gloria A. Gronowicz and Sandra Masur for their help with the EM aspects of this work (3) Polly Chang, Caroline Leake and Annette Hoffman for typing this dissertation, and (4) Cassie Schwartz for her excellent illustrations of the data presented in this dissertation. In addition, I would like

to thank the many other members of the Department of Physiology and Biophysics, who offered help and encouragement. Finally I would like to express my deepest gratitude to the late Dr. Harold Lamport, who advised Dr. Schwartz to accept me as a student in the Department of Physiology and Biophysics and my special thanks to Mrs. Golden Lamport, for her constant support over the years it took me to finish my studies.

TABLE OF CONTENTS

Page

ABSTRACT. v

ACKNOWLEDGMENTS viii

I. INTRODUCTION

1. Background. 1

2. The physiology of ADH action. 3

3. The dual-barrier model for ADH action 4

4. The ADH effect in mammalian renal tubule. 5

5. The separation of ADH effects on water, sodium, and urea permeation. 6

6. The effect of ADH on sodium transport 9

7. The role of cyclic AMP in ADH action. 11

8. Mechanism of cyclic AMP as second messenger 12

9. Cyclic AMP-dependent protein kinase 15

10. Membrane phosphoprotein 20

11. Phosphorylation-dephosphorylation and action of ADH . . . 21

12. Microtubules and microfilament. 25

13. The fine structure of toad urinary bladder epithelium and action of ADH 29

14. The effect of ADH on the epithelium membrane structure. . 33

II. EXPERIMENTAL PROCEDURE

1. Preparation of papillary plasma membrane (PPM). 36

a. Purification of PPM. 36

b. Enzyme marker. 38

c. Electrical microscope study of plasma membranes. . . . 41

Page

II. EXPERIMENTAL PROCEDURE (cont'd)

2. Renal cyclic AMP-dependent protein kinase. 41

a. The standard assay for cyclic AMP-dependent protein kinase activity 41

b. Endogeneous phosphorylation 42

c. Solubilization of membrane-bound protein kinase 42

d. Purification of membrane-bound protein kinase 43

e. Purification of renal cortical and medullary cytosolic protein kinase. 44

f. Cyclic AMP binding assay. 47

g. Autophosphorylation 48

h. Determination of molecular weight of protein kinase . . 49

i. The preparation of regulatory subunit of cyclic AMP-dependent protein kinase. 49

j. G-100 Sephadex gel filtration of regulatory subunit of type II membrane-associated protein kinase (MPK II) . . 50

3. Protein phosphatase. 50

a. Preparation of ^{32}P label phosphoprotein substrate . . . 50

b. Standard assay of protein phosphatase 51

c. Preparation of membrane-bound protein phosphatase . . . 53

d. Preparation of cytosol protein phosphatase. 55

e. Sucrose density gradient centrifugation 57

f. Determination of molecular weight of protein kinase . . 58

4. Phosphorylation-dephosphorylation of renal plasma membrane 58

Page

II. EXPERIMENTAL PROCEDURE (cont'd)

a. Preparation of phosphorylated plasma membrane 58

b. Determination of renal plasma membrane phosphorylation. 59

c. Polyacrylamide gel electrophoresis. 59

d. Cyclic AMP-dependent dephosphorylation. 60

e. Slab gel electrophoresis and autoradiography. 61

III. RESULTS

1. Purification of plasma membrane from the inner medulla of canine kidney (papillary plasma membrane). 62

2. Renal protein kinase 66

a. Subcellular distribution of cyclic AMP-dependent protein kinases from the inner medulla of canine kidney. 66

b. Solubilization of protein kinase from renal plasma membrane. 66

c. Separation of membrane-bound protein kinases. 68

d. Inhibition of membrane-bound protein kinase MPK I and MPK II by heat-stable inhibitor 73

e. Binding of $[^3H]$-cyclic AMP by protein solubilized from papillary plasma membrane 73

f. Autophosphorylation of membrane-bound protein kinase. . 73

g. Purification of membrane-bound kinase MPK II. 76

h. Stability of membrane-bound protein kinase. 76

i. Separation of cytosol protein kinase of renal cortex and medulla 78

Page

III. RESULTS (cont'd)

j. Purification of cytosol protein kinase. 78

k. Substrate differentiation 79

l. Binding of [^{3}H]-cyclic AMP by autophosphorylation of cytosol protein kinases 79

m. Molecular weight of renal cytosol protein kinase CPK II 82

n. Characteristics of canine renal protein kinases 82

3. Renal protein phosphatase. 86

a. Subcellular distribution of protein phosphatase from the inner medulla of canine kidney. 86

b. Solubilization of protein phosphatase from renal plasma membrane. 89

c. Separation of membrane-bound protein phosphatase. . . . 89

d. Purification of membrane-bound protein phosphatase Ma . 91

e. Separation of cytosol protein phosphatase of renal medulla 91

f. Ethanol treatment of cytosol protein phosphatases from renal medulla 91

g. Comparison of cytosol and membrane-bound protein phosphatase 93

h. Separation of cytosol protein phosphatases from renal cortex. 95

i. Purification of cytosol protein phosphatase Pa. 95

j. Characteristics of cytosol and membrane-bound protein phosphatases from canine renal medulla. 96

k. Molecular weights of renal membrane-bound phosphatase Ma and Mb and cytosol phosphatase Pa and Pb 101

Page

III. RESULTS (cont'd)

4. Phosphorylation and dephosphorylation of plasma membrane . 102

a. Phosphorylation of papillary plasma membrane (PPM). . . 102

b. Dephosphorylation of membrane and membrane phosphatase. 104

c. SDS polyacrylamide gel electrophoresis of phosphorylated papillary plasma membrane 107

d. Cyclic AMP-dependent dephosphorylation of papillary plasma membrane 107

e. Autophosphorylation and cyclic AMP-dependent dephosphorylation of membrane protein kinase MPK II 108

f. The nature of cyclic AMP-dependent dephosphorylation of papillary plasma membrane (autoradiography). 110

IV. DISCUSSION

1. Criteria for the involvement of phosphorylated protein in the cyclic AMP-mediated action of ADH. 199

2. Cytosolic protein kinase 202

3. Membrane-bound protein kinase. 204

4. Cyclic AMP-dependent dephosphorylation and membrane-bound protein kinase substrate 206

5. Mechanism of cyclic AMP-dependent dephosphorylation of protein P-50 212

6. Protein phosphatases 219

7. Conclusion 223

REFERENCES 226

LIST OF TABLES

Page

Scheme 1. Purification of Papillary Plasma Membranes 37

Table 1. Specific Activities of Enzyme Markers for Subcellular Fractions from Cells of Canine Renal Medulla 63

Table 2. Relative Specific Activities of Enzyme Markers of Subcellular Fractions of Canine Renal Medulla . . . 64

Table 3. The Activity of Adenylate Cyclase. 65

Table 4. Renal Medulla Subcellular Distribution of Cyclic AMP-Dependent Protein Kinase Activity. 67

Table 5. The Solubilization of Membrane-Bound Protein Kinase. . 69

Table 6. The Activity of Membrane-Bound Protein Kinase. 70

Table 7. Phosphorylation of Renal Medullary Plasma Preparation by Membrane-Bound Protein Kinases MPK I and MPK II . . 72

Table 8. The Inhibition of Membrane-Bound Protein Kinases MPK I and MPK II by Heat Stable Inhibitor. 74

Table 9. Purification of Membrane-Bound Protein Kinase MPK II . 77

Table 10. Purification of Cytosol Protein Kinase CPK II. 80

Table 11. Activity of Renal Cytosol Cyclic AMP-Dependent Protein Kinase with Different Substrates 81

Table 12. Value for Renal Cytosol Protein Kinases CPK I, CPK II and Membrane-Bound Protein Kinase MPK II 85

Table 13. The Comparison of Renal Membrane-Bound Protein Kinase MPK II and Renal Cytosol Protein Kinase CPK II 87

Table 14. Subcellular Distribution of Protein Phosphatase of Renal Medulla. 88

LIST OF TABLES (continued)

Page

Table 15. Solubilization of Protein Phosphatase from Membrane. . 90

Table 16. The Purification of Membrane-Bound Protein Phosphatase Ma 92

Table 17. Ethanol Treatment of Renal Protein Phosphatase 94

Table 18. Renal Cytosol Protein Phosphatase Pa Purification. . . 97

Table 19. The Effect of Divalent Ions on Renal Protein Phosphatase Activity 99

Table 20. The Effect of ATP, ADP, AMP, Pyrophosphate and Phosphate on Renal Protein Phosphatase Activity. 100

Table 21. Km Value and Molecular Weight of Renal Protein Phosphatase. 103

Table 22. The Effect of NaF, Zn^{++} and Theophylline on the Phosphorylation of Solubilized Papillary Plasma Membrane 106

LIST OF FIGURES

Page

Fig. 1. Electrical microscopic study of plasma membrane 111

Fig. 2. Cyclic AMP-dependent protein kinase activity of the solubilized membrane fraction separated on DEAE-Sepharose CL-6B column chromatography 113

Fig. 3. Determination of the molecular weight of the membrane-bound protein kinases MPK I and MPK II by Sephadex G-200 gel filtration. 115

Fig. 4. Binding of [^{3}H]-cyclic AMP by renal medullary plasma membrane fraction separated on DEAE-Sepharose CL-6B column chromatography 117

Fig. 5. Autophosphorylation of membrane-bound protein kinase separated on DEAE-Sepharose CL-6B column chromatography. 119

Fig. 6. Autophosphorylation of membrane-bound protein kinase separated by gel filtration on Sephacryl S-200. 121

Fig. 7. Cyclic AMP-dependent protein kinase activity of renal medullary cytosol separated on DEAE-cellulose DE-52 column chromatography 123

Fig. 8. Protein kinase activity of the cytosol fraction of renal cortex, separated on DEAE-cellulose column chromatography 125

Fig. 9. [^{3}H]-cyclic AMP binding, autophosphorylation and protein kinase activity of cytosol fraction of whole kidney separated on DEAE-cellulose column chromatography. 127

LIST OF FIGURES (continued)

Page

Fig. 10. Determination of the molecular weight of cytosol protein kinase CPK II by Sephadex G-200 gel filtration. 129

Fig. 11. The effect of the concentration of substrate on protein kinase activity 131

Fig. 12. The effect of Mg^{++} on protein kinase activity 133

Fig. 13. The effect of Mn^{++} on protein kinase activity 135

Fig. 14. The effect of Ca^{++} on protein kinase activity 135

Fig. 15. The effect of Zn^{++} on protein kinase activity 138

Fig. 16. The effect of K^{+} on protein kinase activity 140

Fig. 17. pH effect on the activity of cyclic AMP-dependent protein kinase CPK I, CPK II and MPK II 142

Fig. 18. The effect of concentration of cyclic AMP and cyclic GMP on protein kinase activity. 144

Fig. 19. Protein phosphatase activity of papillary plasma membrane fraction separated on DEAE-Sepharose CL-6B column chromatography 146

Fig. 20a. Protein phosphatase activity of the cytosol fraction separated on DEAE-cellulose column chromatography. 148

Fig. 20b. The effect of Mn^{++} and ATP on the cytosol protein phosphatase 148

Fig. 21a. DEAE-cellulose chromatography of ethanol-treated renal cytosol protein phosphatase Pa and Pb 151

LIST OF FIGURES (continued)

Page

Fig. 21b. DEAE-cellulose chromatography of renal cytosol protein phosphatase Pa with ethanol-treated protein phosphatase Pb 151

Fig. 22. Sephadex G-100 chromatography of the ethanol-treated renal cytosol protein phosphatase Pb fraction and cytosolic protein phosphatase Pa fraction. 154

Fig. 23. Phosphatase activity of the cytosol fraction of renal cortex separated on DEAE-cellulose column chromatography 156,157

Fig. 24a. DEAE-cellulose chromatography of the minor peak of cortical phosphatase combined with renal medullary phosphatase Pb 159

Fig. 24b. DEAE-cellulose chromatography of the major peak of renal cortical phosphatase combined with the major peak of medullary phosphatase. 159

Fig. 25. Sephadex G-100 chromatography of the major peak of renal cortical cytosol phosphatase combined with the major peak of renal medullary cytosol phosphatase Pa . 162

Fig. 26. Effect of pH on the activity of cytosol phosphatase Pb 164

Fig. 27. Determination of the molecular weight of membrane-bound protein phosphatase Ma by Sephadex G-100 gel filtration 166

Fig. 28. Determination of the molecular weight of membrane-bound protein phosphatase Mb by Sephadex G-200 gel filtration 168

LIST OF FIGURES (continued)

Page

Fig. 29. Determination of the stokes radii of phosphatases Pa and Pb by Sephadex G-200 gel filtration 170

Fig. 30. Sucrose density gradient ultracentrifugation of protein phosphatases Pa and Pb 172

Fig. 31. The time course of the phosphorylation of papillary plasma membrane. 174

Fig. 32. The time course of the phosphorylation of histone and plasma membrane by protein kinases CPK II and MPK II . 176

Fig. 33. The effect of membrane-bound phosphatase on the phosphorylation of plasma membrane 178

Fig. 34. The effect of NaF and Zn^{++} on the endogeneous phosphorylation of plasma membrane 178

Fig. 35. The time course of dephosphorylation of phosphorylated plasma membrane and histone by protein phosphatases Pa and Ma 181

Fig. 36. SDS gel electrophoresis of phosphorylated plasma membrane 183

Fig. 37. Cyclic AMP-dependent dephosphorylation of phosphorylated plasma membrane, and protein kinases MPK II and CPK II 185

Fig. 38. Polyacrylamide gel electrophoresis of membrane protein kinase MPK II. 187

Fig. 39. Polyacrylamide gel electrophoresis of the holoenzyme and regulatory subunit of membrane protein kinase MPK II 189

LIST OF FIGURES (continued)

Page

Fig. 40. Determination of the molecular weight of the regulatory subunit of membrane-bound protein kinase MPK II by Sephadex G-100 gel filtration. 191,192

Fig. 41. Autophosphorylation of papillary plasma membrane and protein kinases MPK II and CPK II. 194

Fig. 42. Autoradiography of cyclic AM-dependent dephosphorylation of papillary plasma membrane. 196

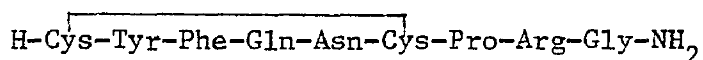
I. INTRODUCTION

1. Background

Water is the major component of living organisms. In vertebrates 45%-80% of body weight is attributable to water, over half of which is within cells. Water serves as the principal physical environment and medium for the transfer of energy as well as for the exchange of chemical substances needed to support the metabolism of living systems. Therefore, it is essential for a living organism to regulate its content of body water in response to changes in osmolality within body compartments. Mammalian organisms are endowed with various mechanisms for the homeostatic regulation of water, such as controlling the intake by thirst (1), but the antidiuretic hormone (ADH) mechanism, which allows variations in osmolality in the body to be in small amplitudes about a well kept mean, is especially important because it saves time and energy which would otherwise have to be spent in continually searching for water.

The discovery by Oliver and Schäfer (2) in 1895 of a pressor effect in mammals upon injection of a bovine pituitary extract constituted the beginning of an intricate trail of research leading to the present active interest in the function and biological activity of vertebrate neurohypophyseal principles and, more specifically, in the chemical and physiological nature of their interaction with target organs. Less than twenty years thereafter, the following additional primary biological effects of neurohypophyseal extracts had been defined: oxytocic (3), milk-ejecting (4), avian depressor (5) and antidiuretic

(6) activities. On the other hand, this early period saw little progress bearing on questions related to the chemical nature of the factor responsible for the biological effects of this extract until 1919 when Dudley (7,8) was able to secure a partial separation of the oxytocic and pressor-antidiuretic activities by means of butanol extraction of an aqueous pituitary solution. It was not until 1949, however, that the first peptide, namely, oxytocin, was obtained in a highly purified state from pituitary extract (9). Pierce and du Vigneaud (10) showed that oxytocin was comprised of eight amino acids and subsequently unraveled the sequence of amino acids by means of desulfurization, performic acid and bromine water oxidation, Edman degradation (11) and partial hydrolysis, concluding that oxytocin was an octapeptide containing a ring formed by a disulfide-bridged pentapeptide unit (12). The isolation of arginine-vasopressin (AVP) from bovine pituitary (13) and lysine-vasopressin (LVP) from porcine pituitary (14), the elucidation of their primary structures, and the synthesis of these hormones were accomplished by du Vigneaud and his associates (15-17). The primary structure of arginine-vasopressin differs from that of oxytocin only in that a phenylalanyl residue replaces the isoleucyl residue at position 3 and an arginyl residue replaces the leucyl residue at position 8. In turn, the structure of lysine-vasopressin differs from that of arginine-vasopressin only in that a lysyl residue replaces the arginyl residue. The primary structure of arginine-vasopressin, the natural ADH in humans, is as follows:



The vasopressins are the natural ADH's in warm-blooded vertebrates,

and in terms of evolution, vasopressins are old hormones that evolved from the closely related parent hormone arginine-vasotocin (AVT), which is the natural ADH found in most cold-blooded vertebrates (17). ADH is present throughout the vertebrate subphylum where it serves primarily to promote water transport across particular epithelial membranes. The role of this hormone is unique in dealing with momentary changes in hydration that must turn on and off rapidly. Accordingly, the half life of ADH in the bloodstream is very brief, of the order of 3-5 minutes.

2. The Physiology of ADH Action

The effect of ADH on transport processes in epithelial tissues has been and continues to be extensively studied. It is clear that understanding the physiological effect of ADH on amphibian epithelia and mammalian renal tubular epithelia will form the basis for analyzing the pathophysiology of clinical disorders of certain membrane transport processes such as nephrogenic diabetes insipidus.

The chief targets for ADH are the renal cortical collecting tubule and the medullary collecting duct (18-20). In amphibia, the hormone increases the permeability of the skin and urinary bladder to water (21-24). In studies of ADH action on frog skin (22-25), toad urinary bladder (19,20,23,26,27) and rabbit cortical collecting tubule (19, 20), it has been shown that ADH affects a limiting water permeability barrier at the apical membrane of the cell. ADH also increases the permeability of amphibian skin and bladder and of mammalian medullary collecting duct but not of cortical collecting tubules to urea (19,20,29). Tissue-labelling methods have shown that the apical cell membrane is the site of the ADH effect on permeability to

urea in amphibia (26). ADH also stimulates the active transport of sodium across amphibian skin and bladder (19,22-24,26) but only exerts a minor effect on the sodium reabsorption from the collecting duct (19, 20,28-30). The nature of the physiochemical change in the membrane responsible for the action of ADH is not yet known.

3. The Dual-Barrier Model for ADH Action

Capraro and Bernini (31) were the first to show that ADH dramatically increases the rate of water movement across the frog skin from the apical to basal-lateral side. Ussing and his colleagues (22,32, 33), in their pioneering studies of water transport across ADH-sensitive epithelia, suggested that water traversed the membrane through aqueous channels by means of bulk flow and that ADH caused the diameter of these channels to increase. They also showed that the hormone appreciably increased the open-circuited transepithelial potential difference (32), the short-circuit current (32) and net apical to basal-lateral flux of sodium (32) and that the net flux of thiourea and acetamide increased in proportion to the rate of osmotically induced water flow in the same direction in ADH-treated frog skin (33). Later Ussing and MacRobbie (25) established that the apical surface of amphibian skin was the rate-limiting step for transepithelial movements of water and solute.

Andersen and Ussing (33) integrated these results into the classical dual-barrier hypothesis for the action of ADH on amphibian epithelia. Apical surfaces were pictured as a series of barriers, a dense outer diffusion barrier that admitted water freely, limited solute penetration and was unresponsive to ADH, and a porous inner

barrier that was sensitive to ADH. It was proposed that the pores in the inner barrier were sufficiently large to permit laminar flow of water and that ADH increased the radii of these pores.

Leaf and his co-workers (27,34-36) subsequently developed convincing arguments in support of the view that a dual-barrier model could account for the effects of ADH on toad urinary bladder. Leaf and Hays (35) concluded that the outer diffusion barrier of the apical surface was rate-limiting to the movement of small solutes whose permeation rates were unaffected by ADH, while the inner porous barrier of this surface limited the transport of water, urea and other small amides. In agreement with the Andersen-Ussing hypothesis, ADH was considered to increase the radii of pores in the inner barrier. On the basis of subsequent experiments involving the combined use of ADH and amphotericin B, Lichtenstein and Leaf (36,37) argued that ADH might also enhance the diffusion of sodium and urea through the dense outer barrier.

4. The ADH Effect in Mammalian Renal Tubules

The effects of ADH on transport processes in mammalian renal tubules, particularly in the case of water permeation, are quite similar to the effects of the hormone on the amphibian skin and toad urinary bladder.

It is believed that ADH exerts the three following actions on the kidney: (a) It increases the permeability of the distal convoluted tubule, the collecting tubule and the collecting duct to water, (b) it also increases the permeability of the collecting duct to urea, but does not affect that of the distal convoluted tubule or cortical collecting tubule, and (c) it directly stimulates sodium reabsorption in the

collecting duct (18,19,38-41).

Release of ADH into the circulating blood plasma increases the permeability of the distal tubule to water in some species (39,41), which then permits fluid entering the distal segment from the loop of Henle to become rapidly isotonic with cortical interstitial fluid and blood plasma. Consequently isotonic fluid enters the collecting ducts, the permeability of which is also increased by ADH; this in turn allows water to diffuse into the hypertonic medullary and papillary interstitium. The final urine is reduced in volume compared with the original filtrate and comes into osmotic equilibrium with the interstitial fluid of the tip of papilla. In the absence of ADH, the hypotonic tubular fluid which enters the distal nephron in large volume is further diluted by reabsorption of solute in the distal tubules and collecting ducts, and water diuresis ensues (18,19,38,39,41).

A number of micropuncture studies have shown that ADH increases the rate of either diffusion and/or osmotic water flow across the mammalian distal tubule and papillary collecting duct (18,28,29,42,43). Grantham et al. (43) have clearly shown that the apical surface of the isolated cortical collecting duct is both the rate-limiting step for water permeation and the barrier whose water permeability is increased by ADH. Grantham (42) has also demonstrated that the hydroosmotic action in cortical collecting ducts is accompanied by increase in the mechanical compliance of the latter. ADH has no effect, however, on the permeability of urea, thiourea, and acetamide in collecting ducts (42-45).

5. The Separation of ADH Effects on Water, Sodium and Urea Permeation

A substantial body of evidence has now accumulated to indicate

that the effects of ADH on water, sodium and urea permeation may be dissociated from each other. ADH affects the so-called tight epithelia (46), which have relatively high electrical resistances in the range 200-2,000 ohm-cm^2 (47). The ADH-dependent increments in water permeation in amphibian skin and toad urinary bladder are accompanied by increases in the permeability of small amides (33,36), in net sodium transport (32,36,48) and transepithelial electrical conductance (35, 36,49). Wright and Sharp (50) have argued that, in toad bladder, a direct action of ADH at lower concentration increases the sodium permeability of the apical membrane, whereas an indirect action of this hormone at higher concentrations elicits the hydroosmotic effect via an increase in the intracellular concentration of cyclic 3',5',-adenosine monophosphate (cyclic AMP).

In isolated segments of the rabbit distal nephron, the effects of ADH on permeation of water, sodium and urea are clearly separable. In cortex (30,42,43,51), outer medulla (51) and papilla (52) ADH increases the permeation of water in a manner similar to that seen in amphibian skin (25) and toad bladder (20) but has no effect on the permeation of urea and small amides (43,44,52) nor on the transepithelial electrical conductance. The effects of ADH on the open-circuited transepithelial potential difference and on net transport of solutes from the lumen are minor (53,54).

Urea and water appear to have separate channels of transport in epithelium because urea does not inhibit water permeation in toad urinary bladder. Levine et al. (55) have shown that phloretin inhibits permeation of urea but not of water in toad urinary bladder (56)

and dogfish kidney (56) both in the presence and absence of ADH, and they have obtained comparable results in toad bladder with tanning agents such as chromate and tannic acid (57). Net transport of sodium and the permeabilities of relative lipophilic species such as ethanol and ethylene glycol are all unaffected by phloretin, tannic acid and chromate either in the presence or in the absence of ADH (55,57).

In amphibian epithelia, permeation of water can be decreased under certain conditions without affecting that of solute. Peterson and Edelman (58) have shown that in isolated toad bladder an elevated concentration of calcium in the bathing medium is antagonistic to the ADH-induced increase in permeability to water while ADH-induced increases in the permeation of urea and in sodium transport are reduced slightly or not at all. In the aquatic toad Xenopus laevis, ADH stimulates sodium transport markedly, but has little or no hydroosmotic effect (59,60). Lipson and Sharp (61) have reported that in toad bladder prostaglandin E_1 enhances and inhibits, respectively, ADH-dependent stimulation of sodium and water transport.

A number of other agents have comparable effects. Chloropromazine irreversibly inhibits the ADH-dependent increment of permeaiblity to water, but increases solute permeation and enhances the ADH-dependent stimulation of sodium and water transport (62). Quinidine (63) inhibits and stimulates, respectively, the ADH-dependent increment in water and net sodium transport. Similarly, cytochalasin B (64,65), colchicine (65), vinblastine (65), podophyllotoxin (65) and cortisol (66) inhibit the hydroosmotic response to ADH, but do not interfere with the stimulatory effects of the hormone on sodium or urea transport.

The general anesthetics such as methoxyfluorane and halothane inhibit ADH-dependent increases in water but not urea permeation (67). This finding is interesting since Pauling (68) suggested that anesthetic agents may decrease the fluidity of membranes by forming ice-like clathrates within the hydrophobic region of the membrane, and recent observations (69) of lipid bilayer membranes by nuclear magnetic resonance spectroscopy have provided direct experimental evidence in support of the assumption that ADH may facilitate water (70) and lipophilic solute (71,72) permeation by increasing the fluidity and/or solubility characteristics of the apical membrane.

A number of agents inhibit sodium transport but not water permeation in toad urinary bladder. The diuretic amiloride (73) and the antimicrobial agent CM-55 (74) interfere with sodium but not water entry at apical surfaces, either in the presence or absence of ADH. Verapamil, a coronary vasodilator and anti-arrhythmic drug, blocks the stimulation of sodium but not water transport produced by adding either ADH or cyclic AMP to basal-lateral surfaces of toad urinary bladder (75). Replacement of sodium by lithium reduces basal and ADH or cyclic AMP-stimulated short-circuit current and sodium transport, but does not affect the hydroosmotic response to ADH (76).

In summary, it is evident from the preceding discussion that in ADH-sensitive epithelia the effect of ADH on water, sodium and urea---or other small amide---permeation may be dissociated.

6. The Effect of ADH on Sodium Transport

As mentioned above, ADH affects the transport of sodium as well as the movements of water and urea. Leaf et al. (34,35,48) observed that

ADH increased the rate of active transport of sodium across isolated toad bladder and proposed that the hormone increases the permeability of the outer (36,37) or inner (35) barrier of the apical surface to sodium. As a result, ADH mediates an increase in the intracellular concentration of sodium, which in turn increases the rate of active pumping through the basal-lateral membrane. This mechanism was presumed to pertain in amphibian skin (22,33,77) as well as in toad bladder (35,36,48,78).

Three observations provided support for this view. First, the apparent total tissue pool of sodium increased with ADH stimulation (77-80). Second, both net sodium transport and the apparent pool of sodium in the epithelium increased as apical concentration of sodium was increased (77,79-81). Third, microelectrode studies showed that the apical membrane had a considerably higher electrical resistance than the basal-lateral surface, and that ADH decreased the resistance of the apical membrane of the epithelium with little change in the resistance of the basilar membrane (82,83).

The apparent saturation of sodium entry across amphibian apical membranes at apical solution concentrations of 30-50 mM (78) is consistent with the possibility that these surfaces contain a saturable transport system (59,85), which may consist of channels or ionophores specific for sodium. In toad bladder, ADH might activate or accelerate the turnover number of these channels or ionophores in apical membranes.

In mammalian cortical collecting tubules, ADH exerts no consistent effect on sodium transport or electrical conductance (53,54). For

example, in rabbit collecting tubules (42,43), ADH has no effect on sodium transport, whereas it increases the movements of water and lipophilic solutes in a manner similar to that observed in toad bladder (35,36). Thus, in ADH-sensitive epithelial cells, water and certain lipophilic solutes appear to traverse the apical plasma membrane via separate pathways in parallel with the route for sodium permeation. In summary, in amphibian epithelia, ADH activates multiple systems; in mammalian cortical collecting tubules, it stimulates the movements of water and lipophilic solutes but not the movement of sodium.

7. The Role of Cyclic AMP in ADH Action

It is well established that the effects of ADH on transport processes in hormone-sensitive epithelia are mediated in accord with the second-messenger hypothesis (86-88). ADH binds to receptors on the basal-lateral surface of responsive epithelial cells and results in the activation of adenyl cyclase, which catalyzes the synthesis of cyclic AMP from ATP. The evidence for this chain of events has been reviewed extensively, both in regard to ADH-receptor interactions (89-93) and control of intracellular levels of cyclic AMP in amphibian epithelia and mammalian renal tubules (86-88,92,94). Intracellular cyclic AMP may involve the two following systems: (a) an intracellular membrane-bound phosphorylation-dephosphorylation system and (b) intracellular microtubule and microfilament structures.

The hypothesis that the physiological effects of ADH on hormone-sensitive epithelia are dependent on cyclic AMP was originally proposed by Orloff and Handler (86-88). It was initially observed that cyclic AMP and theophylline (an inhibitor of phosphodiesterase)

reproduced the effects of ADH on movements of water, sodium and urea in toad urinary (87). It was shown that the content of cyclic AMP in the toad bladder epithelium was increased following exposure to a physiologically active concentration of ADH (95). ADH raised adenyl cyclase activity in tissue slices, cell homogenates and membrane fractions of mammalian renal medulla (90,95-98), but not in those of renal cortex (97). ADH-receptor interaction has been shown to be both specific for tissue and localized to basal-lateral membranes (99). Intracellular activity of adenyl cyclase is also proportional to the degree of hormone binding on receptor sites (89,100).

Orloff and Handler (88) have pointed out that the modulation of ADH effects might occur at one or any combination of four steps: (a) hormone-receptor interaction, (b) adenyl cyclase activity, (c) cyclic AMP degradation mediated by cyclic nucleotide phosphodiesterase and (d) direct interaction of cyclic AMP with cellular mechanisms responsible for changes in membrane transport processes.

8. Mechanism of Cyclic AMP as Second Messenger

The importance of cyclic AMP in the regulation of glycogen metabolism was first recognized in 1957 (101). Cyclic AMP-dependent protein kinase was discovered in muscle in 1968 (102). The latter two findings not only led to elucidation of the pathway by which epinephrine stimulates glycogenolysis in mammalian muscle, but they have also had a profound effect on studies of the mechanism of action of many other hormones. It is apparent that a number of hormones, including ADH raise the level of cyclic AMP in different tissues (103). The activation of protein kinase, at present, appears to be a major mechanism

by which cyclic AMP carries out its function as second messenger (104-109). In the animal cell, the hypothesis that protein kinase mediates all the effects of cyclic AMP is supported by many examples (104-109). No high affinity binding protein for cyclic AMP, other than the regulatory subunit of cyclic AMP-dependent protein kinase and phosphodiesterase (104,108,109), has yet been associated with eukaryotes. Cyclic AMP-dependent protein kinase is found in all mammalian tissues (105-109), and it is present in many of these tissues at approximately the same levels of activity (105-109). These observations support the idea that the enzyme is involved in the control of a number of processes other than glycogenolysis and have led (105-109) to the concept that most if not all of the action of cyclic AMP in mammalian systems may be mediated by the phosphorylation of particular proteins.

Greengard and his colleagues (105) were the first to propose that regulation of protein kinases may be a general mechanism through which the diverse biological effects of cyclic AMP are mediated. According to this hypothesis, a peptide hormone acts on a receptor that is present on the outer membrane of the target cell to cause the generation of cyclic AMP within the cell, which in turn activates intracellular protein kinase to phosphorylate various protein substrates within the cell. Thus the activation of cyclic AMP-dependent protein kinase is not unique to any one particular hormone, and this enzyme is found in a variety of tissues activated by different hormones. Since phosphorylation can affect the biological function of proteins in a variety of ways, this elegant theory provides a unifying concept

that can explain the great diversity in the effects of different hormones (105-106). [The criteria for mediation of an effect of cyclic AMP by phosphorylation of a protein were established by Krebs (107) and refined by Nimmo and Cohen (108).]

Epinephrine acts on liver cells and skeletal muscle cells to accelerate glycogen breakdown and to reduce glycogen synthesis (107-110), the substrates of the protein kinases of their epinephrine-activated target cells being phosphorylase kinase and glycogen synthetase, respectively (106-110). The control of the state of phosphorylation of phosphorylase kinase, phosphorylase b and glycogen synthetase serves to regulate glycogen metabolism in liver and muscle cells (107-109). Cyclic AMP activates phosphorylase b kinase kinase (PBKK) which phosphorylates inactive phosphorylase b kinase (PBK) thus converting inactive (dephospho) PBK to active (phospho) PBK. Active PBK in turn converts phosphorylase b (an inactive entity) to phosphorylase a (an active enzyme).

Larner and colleagues established the existence of two forms, D and I, of glycogen synthetase (111,112). Like the phosphorylase system, the two forms of glycogen synthetase are interconvertible by phosphorylation and dephosphorylation, except that the phosphorylated glycogen synthetase is the less active form (D form). The phosphorylation and inactivation of glycogen synthetase was shown to be catalyzed by the same cyclic AMP-dependent protein kinase which had served to phosphorylate inactive phosphorylase b kinase (113). The discovery of covalent modification of interconvertible enzymes led to the development

of a new concept of metabolic regulatory function--namely, the enzyme cascade system (114-116).

Whether this type of control mechanism also extends to other biochemical and physiological processes is not clear. However, the regulation of enzyme action through the mechanism of interconversion of active and inactive forms by covalent modification can provide rapid switches of enzymatic activity to meet the variable demands of cellular environment. Modification of enzymatic activity through phosphorylation-dephosphorylation is a good example of an intracellular control mechanism of hormonal action.

9. Cyclic AMP-Dependent Protein Kinase

Protein kinases are the enzymes which catalyze the transfer of the γ-phosphate of mucleotide triphosphates to the side chain of amino acid residues in protein substrates. Although the receptor amino acids may be histidine, lysine and arginine (117-119), protein kinases generally catalyze the formation of phosphoester bonds with serine and threonine residues (104,106-109).

Protein kinases have been categorized into cyclic nucleotide-dependent and cyclic nucleotide-independent kinases. The criteria for the classification into these two categories are as follows: Cyclic AMP-dependent protein kinase (CPK) binds cyclic AMP, is stimulated by cyclic AMP and is inhibited by a particular inhibitor protein (120). Although the catalytic subunit of the cyclic AMP-dependent protein kinase does not bind cyclic AMP and is not stimulated by cyclic AMP, the activity of the catalytic subunit is inhibited by the regulatory

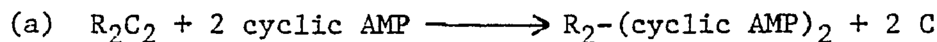
subunit of cyclic AMP-dependent protein kinase and by a heat-stable inhibitor protein (121). None of the effects mentioned above is applicable to cyclic nucleotide-independent protein kinases (121).

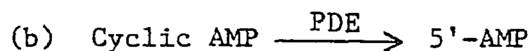
The cyclic nucleotide-regulated protein kinases are stimulated by low concentrations of cyclic 3',5'-purine nucleotides and utilize ATP preferentially as phosphate donor. Cyclic AMP-dependent protein kinases are ubiquitously distributed in eukaryotes (105,122) and have been purified to homogeneity from rabbit skeletal muscle (123) and from bovine heart (124). Cyclic GMP-dependent protein kinases, which have greater affinity for cyclic GMP than for cyclic AMP, have also been isolated from a variety of tissues (125-127).

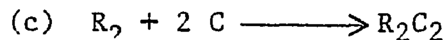
Krebs and his colleagues found that, in addition to phosphorylase kinase, other proteins, e.g. protamine and casein served as substrates for CPK (106-108). Langan found that histone can be phosphorylated by CPK and thereafter histone was adopted as a convenient substrate for studies on CPK _in vitro_ (168).

The CPK holoenzyme was found to be composed of two dissimilar subunits, a regulatory subunit dimer (R_2 monomer R, M_r = 49,000-55,000) which binds cyclic AMP, and two catalytic subunits (C, M_r = 38,000-42,000) which catalyze the phosphotransfer reaction (107-109,123,124, 129-131).

The general mechanism of CPK activation was established (106-110, 123-126,129,130). The interaction events are as follows:


(a) R_2C_2 + 2 cyclic AMP $\longrightarrow$ R_2-(cyclic AMP)$_2$ + 2 C


(b) Cyclic AMP $\xrightarrow{PDE}$ 5'-AMP


(c) R_2 + 2 C $\longrightarrow$ R_2C_2

(a) The cyclic AMP binding to regulatory subunit (R) causes dissociation of inactive holoenzyme (RC) to yield an active catalytic subunit (C).

(b) An increase in the concentration of cyclic AMP activates phosphodiesterase (PDE) which, in turn, converts the cyclic AMP to 5'-AMP. Thus, the concentration of cyclic AMP rapidly falls to a very low level, and free regulatory subunit R is produced when the bound cyclic AMP dissociates.

(c) The free R rapidly reassociates with C thereby reforming the inactive holoenzyme.

Thus, the concentration of cyclic AMP controls the amounts of free catalytic subunit which, in turn, brings about the phosphorylation of protein substrates.

A cyclic AMP dependent protein kinase can be free in the cytosol or bound to cell membranes. In addition cytosol CPKs have been separated into two major fractions in any tissue extracts by ion-exchange chromatography (106-109,133-135). These fractions have been identified and referred to as type I and type II (130-133). These two types of enzymes can be further distinguished by the ability of histone or high salt concentration to alter their dependence on cyclic AMP (132-135). Type I protein kinases elute with 0.08-0.10 M NaCl from DEAE cellulose and their subunits reassociate slowly following cyclic AMP-induced dissociation. In contrast type II protein kinases elute from DEAE-cellulose with 0.15-0.2 M NaCl, dissociate

slowly upon addition of histone or salt, and reassociate rapidly following cAMP-induced dissociation (108,133-135). All tissues examined contain both types of protein kinase, although the proportion of each are different depending upon tissue and species (108,133,134)

The best studied type I protein kinase was isolated and purified from rabbit skeletal muscle by Kreb and colleagues (107,123). Later Rosen and associates purified type II protein kinase from bovine cardiac muscle (124,129,131). There are both similarities and differences in the biochemical properties of these two types of protein kinase. Each enzyme has an $S_{20.W}$ value of 7.0. Each enzyme binds 2 mole of cyclic AMP per mole of enzyme and dissociates in the presence of saturating concentration of cyclic AMP into a dimeric regulatory subunit-cyclic AMP complex and two catalytic subunits. The isolated subunits recombine, resulting in the formation of the original holoenzyme. These two isozymes may have similar or identical catalytic subunits (Mr=40,000) and differ only in their regulatory subunits (135) (e.g. with respect to their sedimentation constants and mobility on SDS gel electrophoresis). The regulatory subunit of skeletal muscle CPK has a lower molecular weight (M_r = 49,000) than the analogous subunit of carduac muscle CPK (M_r = 55,000) (135). The regulatory subunit of the heart kinase is rapidly phosphorylated by Mg^{++}ATP, but this does not occur in the case of the skeletal muscle enzyme. Mg^{++}ATP only binds to type I enzyme, and thereby lowers its affinity for cyclic AMP. Mg^{++}ATP increases the dissociation constant for cyclic AMP about 10 fold and decreases the Hill coefficient; however, in the case of the heart kinase, Mg^{++}ATP decreases the dissociation constant for cyclic AMP 5-6 fold and in-

creases the Hill coefficient (129,133,135). Different concentrations of cyclic AMP are required to dissociate the skeletal and heart muscle enzymes (129,130,135). The presence of Mg^{++}ATP increases the concentration of cyclic AMP required to dissociate the skeletal muscle enzyme but decreases the concentration of cyclic AMP necessary to dissociate the heart enzyme (129,135).

The type II isozyme catalyzes the phosphorylation of its own regulatory proteins (2 moles of phosphate per mole of holoenzyme) (136,137); the phosphorylation site has been identified as phosphoserine (136). The self-phosphorylation reaction is rapid both in the presence or absence of cyclic AMP. When the reaction rate is slowed by lowering the concentration of ATP to 0.06 mM, a two fold stimulation of the self-phosphorylation reaction by cyclic AMP occurs (129,136,137). The phosphorylation of the type II isozyme reduces its dissociation constant for cyclic AMP from 2.8 μM to 0.4 μM and reduces the rate of reassociation of the dissociated regulatory and catalytic subunits (137). The phosphorylated type II isozyme can be dephosphorylated by a phosphoprotein phosphatase (Mr=30,000) purified from bovine heart (138). The dephosphorylation reaction requires the addition of cyclic AMP, which appears to interact with the substrate (138). Whether these isozymes have different physiological roles remains to be determined.

The cyclic AMP-dependent protein kinase have a broad substrate specificity (e.g., histone, casein, phosphorylase kinase, glycogen synthetase, lipase, etc.) (104,107-109). The basic residues on the N-terminal side of the phosphorylated serine residue may be important determinants of the substrate specificity of cyclic AMP-dependent pro-

tein kinases (104,107-109).

Membrane-bound protein kinases have different properties than cytosol protein kinases. Uno et al. have purified a membrane-bound protein kinase from a particulate fraction of bovine cerebral cortex enriched in synaptic membranes (139). They compared this kinase to a type II cytosolic protein kinase from bovine heart and found that the holoenzyme and the regulatory and catalytic subunits differed from the corresponding components of the cytosolic enzyme with respect to molecular weight, isoelectric point subunit recombination, catalytic properties and immunological properties. As expected the regulatory subunit of the membrane-bound enzyme recombined with and inhibited its own catalytic subunit. Moreover the regulatory subunit from either the brain or heart cytosol protein kinase could combine with and inhibit the catalytic subunit of either brain or heart cytosolic enzyme. However, the regulatory subunit of the membrane-bound protein kinase could not inhibit the catalytic subunit of either cytosolic enzyme, nor could the regulatory unit of either cytosolic enzyme inhibit the catalytic subunit of the membrane-bound enzyme (139).

Presumably, the specific properties of the membrane-bound protein kinase reflect a specific functional role for this enzyme.

10. Membrane Phosphoprotein

The interconversion of key enzymes between active and inactive forms through cycles of covalent modification is an important control mechanism involved in the regulation of many biological processes (108,109, 140-144). In eukaryotes, phosphorylation has been found to be one of the most important metabolic regulatory mechanisms involved in mediating

the function of a number of hormones (e.g. ACTH, glucagon and epinephrine) (103). Because many important cellular functions involve membrane phenomena (e.g. water and solute permeability and transport processes, transduction of neural signals, and secretory processes) and because biological membranes are a rich source of protein-bound phosphate, a number of studies have been directed at the possible physiological significance of membrane phosphoprotein of erythrocytes, cells of secretory tissues, synapses and hormone-sensitive epithelia (108,109).

11. Phosphorylation-Dephosphorylation and Action of ADH

In the case of tissues that are sensitive to vasopressin, it is possible that cyclic AMP regulates transport by activation of a cyclic AMP-dependent protein kinase. This hypothesis implies that increased protein phosphorylation alters apical plasma membrane permeability. In ADH-sensitive tissue of frog bladder (145), toad bladder (146), and bovine renal medulla (94), cyclic AMP-dependent protein kinase activity has been demonstrated using exogeneous substrates. The soluble enzyme from bovine medulla was also found to be capable of phosphorylating a denatured plasma membrane preparation from the same tissue (94).

In studies of apical and basolateral membrane preparations from bovine renal papillary cells, vasopressin-sensitive adenyl cyclase activity was found only in the basolateral membranes and cyclic AMP-dependent protein kinase activity as detected by phosphorylation of the membrane fraction itself was found only in the apical membranes (99). These findings led to the suggestion that apical membrane phosphorylation might underlie the increased apical membrane permeability that characterizes the hydroosmotic action of ADH.

It has been proposed that cyclic AMP alters apical membrane permeability by enhancing protein dephosphorylation (147-150). This latter hypothesis is based on studies in the urinary bladder of the toad, Bufo marinus (147,148). These studies involved labeling of bladder proteins with ^{32}P-phosphate, exposure of the labeled bladders to ADH, separation of the bladder (presumably epithelial cell membrane) proteins to ADH using SDS gel electrophoresis followed by radioautographic comparison of the ^{32}P labeling of proteins derived from hormone treated versus control bladders. The most prominent hormone effect noted was a decrease in the ^{32}P content of a protein of molecular weight approximately 50,000 which was referred to as protein D. A similar decrease in ^{32}P recovered in protein D was observed when toad bladders were exposed to monobutyryl cyclic AMP (147,148). This change preceded the increase in sodium transport measured simultaneously in paired control hemibladders (147-149). Cyclic AMP lowered the phosphate content of protein D by increasing its rate of dephosphorylation, an effect attributed to enhancement by cyclic AMP of the phosphoprotein phosphatase activity of the bladder (147-149). Agents that increased sodium transport (ADH, cyclic AMP, theophylline, adenine, PGE_1 and manganese chloride) also decreased the amount of phosphate remaining in protein D (147). Zinc chloride inhibited sodium transport and increased the phosphate content of the protein. Change in protein phosphorylation did not consistently correlate with water permeability changes (149,150). These results were interpreted by the authors as compatible with the view that cyclic AMP regulates sodium transport by decreasing the phosphorylation of a protein (M_r = 50,000) through activation

of a protein phosphatase (148).

Recently, Malkinson (151) et al. reported the presence of a phosphoprotein (M_r = 50,000) the dephosphorylation of which was stimulated by cyclic AMP in all vertebrate tissues examined, including toad bladder. When tissue homogenates containing this phosphoprotein were labeled with a photoaffinity analogue of cyclic AMP the phosphoprotein and the cyclic AMP analogue comigrated on electrophorsis, a finding which led these workers to suggest that the phosphoprotein was the phosphorylated regulatory subunit of a protein kinase. This comigration, however, does not constitute proof of this suggestion. For example, in the human erythrocyte membrane (152), a cyclic AMP binding protein and a phosphoprotein, both of M_r = 48,000 comigrate on gel electrophoresis, but the two molecules are separable by extraction with a non-ionic detergent.

Liu & Greengard (153) showed that treatment of toad bladders with aldosterone decreases the incorporation of phosphate into the 50,000 dalton protein and enhances its rate of dephosphorylation in the presence or absence of cyclic AMP. They suggested that aldosterone may regulate sodium transport by this mechanism. In rat, several other steroid hormones were shown subsequently to decrease the incorporation of phosphate into a soluble protein (M_r = 50,000) in their respective target tissues (154).

The 50,000 daltons protein the dephosphorylation of which appears to be mediated by cyclic AMP was first referred to as protein D (147) and later as P-50 (151) and as SCARP, the latter name designating steroid and cyclic AMP-regulated phosphoprotein (154). Liu and Greengard

extended their studies by examining toad bladders in which sodium transport was stimulated by aldosterone (154) and in which the metabolism of SCARP was studied in a membrane preparation or in cell sap. They found that more phosphorylated SCARP was present in the membrane preparation obtained from aldosterone-treated tissue than in the preperation obtained from control tissue. Incubation with aldosterone resulted in membranes that removed phosphate from SCARP more rapidly than untreated control membranes. The same relationship held in the presence of cyclic AMP (153). The effect of aldosterone on SCARP phosphatase activity, like the aldosterone effect on short circuit current, was blocked by spironolactone and by inhibitors of protein synthesis, but not by nonmineralocorticoid steroid hormones such as testosterone (153). Thus, aldosterone which stimulates sodium transport without altering cyclic AMP levels in toad bladder stimulates the activity of SCARP phosphatase (152).

Although aldosterone by itself has no effect on the permeability to water in the toad bladder, it alters this permeability response to ADH (155). Aldosterone alone does not affect tissue cyclic AMP levels in toad bladder epithelium, but aldosterone does greatly enhance the cyclic AMP response to vasopressin (153). This is associated with inhibition by aldosterone of both low K_m and high K_m soluble cyclic AMP phosphodiesterase activity (156), a phenomenon which in turn may explain why aldosterone increases SCARP dephosphorylation because inhibition of phosphodiesterase serves to maintain or to increase intracellular cyclic AMP levels.

The effect of steroid hormones on SCARP appears to be rather general. Liu and Greengard (154) found that the effects of testosterone on seminal vesicle and prostate, of estradiol on the uterus, and of cortisol on the liver, in part, resemble those of aldosterone on the toad bladder. Target tissues treated with testosterone, estradiol and cortisol exhibit reduced rates of phosphorylation of an endogeneous protein of M_r=50,000. This steroid hormone effect was seen only in the appropriate target tissue and was blocked by cycloheximide (154). The steroid hormone treatment reduced not only the rate of phosphorylation but also the amount of phosphorylation of SCARP in toad bladder, even under conditions in which phosphoprotein phosphatase activity was completely inhibited by zinc. Steroid appears to regulate the amount of SCARP or the ability of SCARP to become phosphorylated. If we assume that protein P-50 is the regulatory subunit of a type II protein kinase, then SCARP also would be the regulatory subunit of a protein kinase. The reduction in phosphorylation induced by steroids would not be expected to enhance the response of the kinase to cyclic AMP because it has been observed that the enzyme is more sensitive to activation by cyclic AMP when the regulatory subunit is phosphorylated (151).

12. Microtubules and Microfilament

A number of workers have proposed that microtubules and microfilaments are involved in the action of cyclic AMP in vasopressin-sensitive tissue (157,158). Microtubules have been recognized to be associated with several types of cell movement (159), and with the translocation of cell constituents. Microtubules may be involved indirectly in the

generation of cellular movements concerned with the maintenance of cell shape, or they may actually participate in processes of mechanico-chemical transduction analogous to those occurring in muscle (159). Evidence for a role of microtubules in the cellular action of ADH is based on the observation that microtubule-disrupting alkaloids such as colchicine, vinblastine or podophyllotixin inhibit the hydroosmotic response to ADH and exogeneous cyclic AMP in the amphibian urinary bladder (65,158). In contrast to the inhibitory effects of the alkaloids on vasopressin and cyclic AMP-induced water movement, colchicine, vinblastine and podohyllotoxin were found to have no effect on the active transport of sodium across the bladder after a 4 hour exposure of the toad bladder to these alkaloids (157). This finding indicates that the effect os vasopressin on water and sodium movement involves separate cellular mechanisms, a conclusion which is in accord with the fact that the effect of vasopressin on water movement is calcium-sensitive, whereas the effect of the hormone on sodium transport is not (157). Taylor et al. also have evidence that the inhibitory effect of colchicine on vasopressin induced water movement depends on the interaction of colchicine with tubulin in the target cells (65,157). Their studies indicated that tubulin accounts for an appreciable quantity of the soluble protein of bladder epithelial cells.

On electron microscopic examination of untreated bladders, microtubules appeared to be randomly distributed in the cytoplasm of the granular cells; in the colchicine-treated bladder (157) the microtubules in the granular cells were reduced in number. These observations suggest that intact cytoplasmic microtubules are required for the cellular action of vasopressin, probably in steps subsequent to the generation of cyclic AMP.

Dousa et al. examined the influence of ADH and cyclic alkaloids had no effect on the basal permeability to water across the tissue. However, the vasopressin-stimulated increase in water movement was reduced in a concentration dependent manner in hemibladders that had been exposed to each of these agents. The non-hormonal hydroosmotic response elicited in the amphibian bladder by hyperosmolar medium was not inhibited by colchicine (65). Colchicine and vinblastine also blocks the anti-diuretic response to exogeneous ADH in the unanestetized rat (160) and diminishes the antidiuretic response to ADH in the anesthetized rat (160). These alkaloids, in the doses employed, have no major effect on other renal functions (159-161). Intraperitoneal colchicine given to rats over several days reduces the antidiuretic effect of exogeneous vasopressin without significantly affecting solute excretion (157,160) vinblastine has the same effect. Neither of these agents inhibits adenylate cyclase, phosphodiesterase or cyclic AMP-dependent protein kinase *in vitro* (157,158).

While colchicine and vinblastine in low concentrations have no effect on the activities of enzymes postulated to participate in the cellular action of ADH *in vitro* (158,160), vinblastine in the same low concentration range blocks microtubule polymerization in cell-free renal medullary extracts (162-164).

In contrast to their inhibitory effects on vasopressin-induced and cyclic AMP-induced water movement, colchicine, vinblastine and podophyllotoxin were found to have no effect on the rate of microtubule polymerization in the renal medulla (163). Vinblastine and LiCl both block vasopressin action *in vivo*, and diminish the rate of microtubule polymerization (162,163). An increase in intracellular calcium concentration also reduces microtubule polymerization and decreases the

target organ response to vasopressin. In the same way guinidine reduces microtubule polymerization by 50% and also inhibits the hydroosmotic response to ADH in the toad bladder. However, ADH has no direct effect on the polymerization of microtubules, nor do increases in intracellular cyclic AMP levels affect the polymerization of microtubules (158,163-165). Although cyclic AMP dependent protein kinase has been found to phosphorylate tubulin from brain and phosphorylation appears to facilitate microtubule assembly, tubulin is not a very good substrate for protein kinase (158,165). It appears that cyclic AMP dependent-protein kinase can phosphorylate tubulin or microtubule associated protein, but it is unknown whether such phosphorylation influences microtubule function in renal medullary tissue. The available information does not as yet identify any relationship between cyclic AMP dependent phosphorylation and microtubular function in the mediation of the cellular action of vasopressin. At the present time it appears that the two processes are not immediately related but rather that they are involved in an independent and parallel way in the overall coordinated response to the hormone.

There are also several points to be determined before we can assess the precise role of microtubules in the ADH-induced increase in water permeability. The inhibitory action of colchicine and related agents on the permeability response to vasopressin and cyclic AMP are in all probability referable to an effect of these agents on cytoplasmic microtubules, because little if any tubulin is associated with the plasma membrane of toad bladder or rat medullary epithelial cells (162). Prior to the studies implicating microtubular function in the membrane

action of vasopressin, it has been demonstrated that in the presence of the hormone there was an increase in the frequency with which cytoplasmic granules moved to and fused with apical plasma membranes of toad bladder epithelial cells, and it was postulated by Masur et al. (166) that this enhanced addition of granule membrane to cell membrane may account for the characteristic ADH-induced increase in apical membrane permeability. This hypothesis is in accord with the fact that, in other tissues, microtubules are associated with intracellular movement of secretory granules and exocytosis.

13. The Fine Structure of Toad Urinary Bladder Epithelium and Action of ADH

Over the last twenty years, the toad urinary bladder has been proved invaluable as a model system for the study of transport systems and the mechanisms whereby hormones such as vasopressin and aldosterone modulate these systems. Since the epithelium of toad urinary consists of single layer and four morphologically distinct cell types, there is a strong possibility that these diverse cell types differ with respect to their transport physiology and/or with respect to their response to hormones. Before we conclude our discussion of the mechanism of action of ADH on renal tissue, it is important to consider the action of ADH on the amphibian urinary bladder. During the past 25 years it has been established that the apical border of the vasopressin-sensitive epithelial cell represents tha rate-limiting barrier for permeability to water and therefore constitutes the final site of action of the antidiuretic hormone.

There are four types of cells in the mucosal epithelium, namely, granular, mitochondria-rich, goblet and basal cells (167,168). Although the earlier studies showed and only three of these cell types are in contact with urinary surface, more recent observations with EM scanning showed that mitochondria-rich cells have dense, thick, rounded microvilli which are arrayed in relative small, narrow areas between the apical borders of granular cells. The granular cells are polygonal flat and contain microvilli that vary in length and density from cell to cell. The granular cell junction forms a ridge which seems to consist of an elevated sequence of microvilli adhering to each other (168).

The granular cell has a markedly higher content of the characteristic lysosomal hydrolase acid phosphatase and cathepsin B_1 than that found in the mitochondria-rich cell (169). Incubation of granular cells with vasopressin elicits a significant increase of water and calcium content and an increased extracellular release of lysosomal enzymes as compared to untreated control cells. In the case of mitochondria-rich cells treated with vasopressin, cell calcium declines slightly, but no significant increase in water content or extracellular hydrolase activity has been detected in comparison with paired control cells (167).

Osmotic water flow is routed through the epithelial cells rather than through the apical tight junction (167,170). Since both radioactive tracer (170) and electrophysiologic (35,171) data indicate that the effect of vasopressin on sodium transport is mediated at the apical

plasma membrane, and since the apical effect of vasopressin increase the volume of the granular cell resulting in bulk water flow limited to these cells, it was assumed that the hormonal effect on net sodium transport is also limited to the granular cell (167). The mitochondria-rich cells, as this designation indicates, contain a higher concentration of mitochondria than do the granular cells; this suggests that they subserve functions coupled to oxidative metabolism. Using scanning and transmission electron microscopy, Danon et al. (168) discovered that cell counts reveal Bufo marinus toad bladder epithelium contains 3 or 4 granular cells to 1 mitochondria-rich cell.

Handler et al. (172), employing the technique developed by Scott et al. (173) to separate the mitochondria-rich and granular epithelial cells of toad urinary bladder, showed that mitochondria-rich cells and granular cells both contain vasopressin-sensitive adenylate cyclase at the same levels of specific activity. Also both types of cells were found to have cyclic nucleotide phosphodiesterase activity. At low substrate concentrations, the enzyme in granular cells is more active than the enzyme in mitochondria-rich cells (172).

After immunofluorscent localization of cyclic AMP in toad bladder epithelial cells, Goodman et al. (174) found increased levels of cyclic AMP in all epithelial cell types within 2 min following exposure to vasopressin.

Incubation of granular cells results in release of lysosomal hydrolase, acid phosphatase and cathepsin B_1 (169). The physiological significance of acid proteinase released from granualr cells is unclear;

however, proteinase inhibitor which suppresses the activity of cathepsin B_1 selectively antagonized the action of hormone on water permeation. It was suggested that alteration of the intracellular level of Ca^{++} and of lysosomal hydrolase activity in granular cells may be related to ADH-induced increases in permeation (169).

Recently Zusman *et al.* (175) showed that vasopressin stimulates prostaglandin E_2 (PGE_2) biosynthesis in the toad urinary bladder by a mechanism that does not depend on the increase in intracellular cyclic AMP level. Neither cyclic AMP nor theophylline stimulates PGE biosynthesis, although both of the agents mimic the effect of arginine vasopressin on water permeabilty. Prostaglandins E_1 and E_2 inhibit the water permeation response of the toad urinary bladder to vasopressin and to theophylline but do not inhibit the response to cyclic AMP (176, 177). Thus inhibition by PGE of the response to ADH is mediated at the level of adenylate cyclase and appears to be a built-in counter regulatory mechanism for modulation of the hormonal action.

In general, the apical and basolateral regions of the epithelial cell membrane may be distinguished by several ultrastructural and cytochemical characteristics. Thus, the apical plasma membrane of toad bladder epithelium is covered by an outer coat of "fuzz" or glycocalyx (178). This glycocalyx contains sugar and protein in abundance as indicated by staining characteristics and susceptibility to proteolytic enzymes (179). It binds cationic dyes (180) as a consequence of its anionic surface glycoprotein (181). Pisam and Ripoche (182) found that the glycoprotein coating the apical surface of the frog urinary bladder

epithelial cell differs from that which lines the lateral and basal surfaces. After dissociation and isolation of the epithelial cells, the material previously confined to the apical surface invaded progressively the open tight junction, then the lateral membrane and finally the basal membrane; ultimately the whole cell surface was entirely enveloped by the apical material. Thus, in the intact epithelial sheet the tight junction appears to play a role in confining one or more apical surface constituents to the luminal aspect of the epithelium and, in this manner, the tight junction may contribute to maintaining the specialization of vasopressin-sensitive epithelia (where the apical membrane must be maintained as a hormonally modifiable permeability barrier).

14. The Effect of ADH on the Epithelium Membrane Structure

Chevalier et al. (183), using the freeze-fracture technique to study the fine structure of the plasma membrane of frog urinary bladder epithelial cells at rest and after oxytocin treatment, found that the external or cytoplasmic leaflet (A face) of the apical membrane of epithelial cell is characterized by (1) a markedly reduced number of membrane-associated particles as compared to the A face of the lateral membrane of this cell and (2) the presence of numerous areas of clustered particles only in the membranes derived from bladders that were treated with hormone. Wade et al. (184) confirmed these findings in the toad urinary bladder and in addition noted (1) that the majority of particles are located on the fracture face B (outer membrane face) in the case of the luminal membrane whereas the majority of particles are located on the

fracture face A (inner membrane face) in the case of the basal membrane and (2) that the A and B fracture faces of the basal membrane have a similar distribution of particle size in the case of the luminal membrane whereas the B face has particles which are generally larger than those found on the A face. This structural specialization of the granular cell luminal membrane may be related to the permeability functions and to the hormonal regulation of these functions. Kachadorian et al. (185) observed a vasopressin-induced and a cyclic AMP-induced aggregation of the intramembranous particles in orderly linear arrays at multiple sites in the luminal membrane specifically of the granular cell, specifically in response to hormonal challenge of the serosal aspect of the bladder, independently of the presence of an osmotic gradient. The degree of vasopressin-induced particle aggregation in terms of the number of cluster per unit area of membrane or the cumulative area of membrane occupied by clusters is closely correlated with the induced changes in osmotic water flow. Thus, the aggregates per se may be of physiological significance in the mechanism of action of vasopressin.

In further study of this phenomenon, Kachadorian et al. (186) found that the aggregation response first became detectable after 2.5 minutes of exposure to ADH when osmotic water flow is in the process of initiation. The aggregation phenomenon was found to be maximal at 30 minutes when water flux is also maximal; aggregation was dramatically diminished 5 minutes after hormone washout when the induced osmotic water permeability is also almost completely attenuated. These data provide strong support for the concept that the aggregation response to ADH is related

to the ADH-induced increase in membrane permeaiblity.

Evidence obtained from lipophilic probes of membrane of amphibian bladder (187-188) and mammalian kidney (189) indicate that vasopressin acts to enhance the disorder (i.e., to increase the fluidity) of membrane lipids. Such increments in membrane fluidity may contribute to the redistribution and aggregation of lectin-binding protein detected at external surface of bladders treated with vasopressin (182). This observed redistribution of membrane-associated protein on the apical surface of frog urinary bladder epithelium after oxytocin treatment (183-186) is in all probability related to the hormone-induced particle aggregation discussed above.

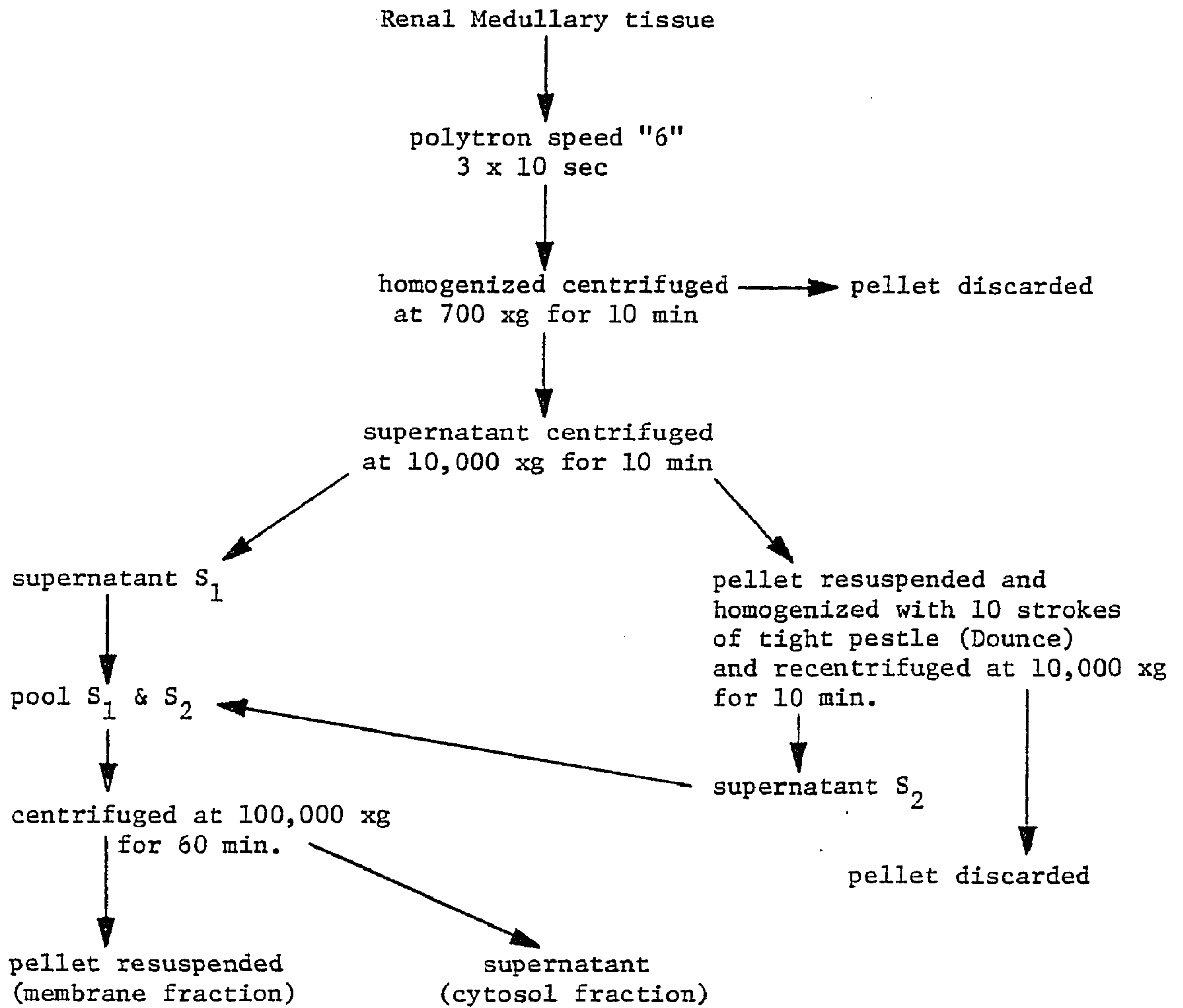
II. EXPERIMENTAL PROCEDURE

1. Preparation of Papillary Plasma Membrane (PPM)

a. Purification of PPM

Dog kidneys were excised from freshly slaughtered animals within 1 hour after death. The inner medulla was separated from the cortex. 40-80 g of medullary tissue were collected. The medullary tissue was cut into small pieces of about 1 mm^3 with a pair of scissors and put in a Tris-glycine buffer (10 mM Tris·HCl, 0.28 M glycine, pH 7.4), with a ratio of tissue weight to total volume of 1:3. The tissue was then treated according to the scheme outlined in Fig. 1. First, the tissue was homogenized (in a Brinkman Polytron at speed "6") for 3 successive intervals each of 10 seconds duration. The homogenate was then subjected to 15 strokes with a loose pestle in a Dounce homogenizer, filtered through a layer of cheese cloth, rehomogenized with 15 strokes of a tight fitting pestle, and centrifuged at 700 xg for 10 min. The pellet was discarded. The 700 xg supernatant was further centrifuged at 10,000 xg for 10 minutes to yield the supernatant S_1. The pellet was resuspended in the same relative volume of buffer (see above), and subjected to 10 strokes with a tight pestle in a Dounce homogenizer and recentrifuged at 10,000 xg for another 10 minutes. The new supernatant S_2 was pooled with S_1 and the mixture subjected to centrifugation at 100,000 xg for 60 min (Beckman swinging bucket SW 25 rotor). The resulting supernatant was labeled "cytosol fraction," and the pellet resuspended with a same volume of Buffer A (20 mM Tris·HCl, pH 7.0; 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 20 mM KCl) was labeled "membrane fraction."

Purification of Papillary Plasma Membranes



b. Enzyme Markers (used to characterize the subcellular fractions)

(1) 5'-Nucleotidase Assay according to Solyom and Trans (191)

The enzyme was assayed at 37°C in a total volume of 0.5 ml containing 5 mM adenosine monophosphate (AMP), 10 mM $MgCl_2$, 50 mM Tris-glycine buffer adjusted to pH 9.1 with NaOH, and 50 μg of protein. The reaction was stopped after 20 minutes of incubation by the addition of 2.5 ml of 8% trichloroacetic acid (TCA). The solution was filtered through Whatman No. 42 paper and a 1.8 ml aliquot of the filtrate was added to 1 ml of freshly prepared "binding reagent" solution (2.39 g of ferrous sulfate in 100 ml of 1% NH_4 molybdate in 2 N H_2SO_4) for the measurement of inorganic phosphate released by the method of Fisk & Subbarow (192).

(2) Glucose-6-Phosphatase Assay according to Hubscher and West (193)

The activity of glucose-6-phosphatase was assayed at 37°C in a total volume of 0.2 ml containing 20 mM Tris·HCl buffer (pH 7.0), 4 mM EDTA, 50 mM glucose-6-phosphate, and 50 μg of protein. The reaction was stopped by addition of 50 μl of 30% TCA. The mixture was then centrifuged and the supernatant was removed and assayed for inorganic phosphate by the method of Fisk & Subbarow (192).

(3) Sodium-Potassium ATPase

The activity of Na^+-K^+-ATPase was assayed by incubation of the enzyme at 37°C in a total volume of 0.25 ml containing 50 mM Tris HCl (pH 7.6), 4 mM $MgSO_4$ with or without 1 mM ouabain, 48 mM NaCl, 24 mM KCl, 1 mM ATP, and 50 μg of protein. The reaction was allowed to proceed for 30 min and then stopped by addition of 50 μl 60% TCA followed by centrifugation. An aliquot of supernatant was kept and

the amount of inorganic phosphate liberated was determined by the method of Fish & Subbarow (192).

(4) Bicarbonate-Activated ATPase

HCO_3^--ATPase was determined by incubating the enzyme for 30 minutes at 37°C in a total volume of 0.25 ml containing 50 mM Tris·HCL buffer (pH 7.2), 4 mM $MgSO_4$, 1 mM ouabain, 5 mM ATP, with or without 25 mM sodium sulfite and 50 μg of protein. The reaction was stopped by addition of 50 μl of 60% TCA. The amount of organic phosphate liberated was determined by the method of Fish and Subbarow (192). The activity in the absence of sulfite was taken as the Mg^{++} ATPase activity and the difference between the activity in the presence of sulfite and the activity in the absence of sulfite was taken as the HCO_3^--ATPase activity (99).

(5) Succinate Dehydrogenase Activity Assay according to Gibbs and Reimer (194)

The assay of the enzyme activity was carrier out at 37°C. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1% 2,6-dichloroindophenol, 50 mM of sodium succinate, and 50 μg of protein in a total volume of 200 μl. The reaction was allowed to proceed for 5 min and then was stopped by addition of 50 μl 30% TCA and 1 ml of ethyl acetate with shaking for 1 or 2 minutes, centrifugation for 2 min. The supernatant was recovered and the optical density of the fumarate generated was read at 490 μm.

(6) Adenylate Cyclase Assay

The adenylate cyclase activity of the isolated membrane preparations was assayed according to the method of Bär and Hechter (195).

Under standard assay conditions, 30-55 μg of membrane protein were incubated at 37°C for 5 min in a total volume of 50 μl containing 50 mM Tris HCl (pH 7.5 at 37°C), 0.2 mM ATP, 1-2 μCi [α-^{32}P]ATP, 10 mM $MgCl_2$, 1 mg/ml creatine phosphokinase, 25 mM creatine phosphate, 0.5 mM cyclic AMP, 1 mM EGTA and 1 mg/ml bovine serum albumin.

The reaction was started by addition of protein and terminated by addition of 5 μl of a solution containing 20 mM each of ATP, AMP and cyclic AMP in 0.2 M EDTA (pH 7.0) and the tubes placed on ice. When the time course of cyclic AMP production was determined, the total reaction volume was increased to 100 μl. After the start of the reaction, 10 μl aliquots were removed from the reaction mixtures and transferred to tubes containing an equal volume of an ice-cold solution containing 4 mM each of ATP, AMP and cyclic AMP in 40 mM EDTA (pH 7.0). The contents of the tubes were mixed thoroughly and the tubes placed on ice.

Labeled nucleotides were separated by application of 5 μl aliquots of the reaction mixture to polyethyleneimine (PEI) impregnated cellulose TLC plates, which were then developed in 0.25 M LiCl. After the plates were dried, cyclic AMP, ATP and AMP spots were located under UV illumination. These were then cut out and counted in toluene scintillation fluid [4.0 g PPO (2,5-diphenyloxazole) and 0.2 g POPOP [1,4-bis(2-(5-phenyloxazole))-benzene/l] with a liquid scintillation spectrometer. The ratio of cyclic AMP associated radioactivity to total radioactivity was used as an estimate of the conversion of substrate to cyclic AMP and the specific activity of the enzyme was expressed as pmole cyclic AMP produced per mg protein per min.

To test the adequacy of the regenerating system, aliquots of the reaction mixtures were applied to PEI-impregnated cellulose TLC plates and chromatographed in 0.5 M nucleotides visualized as described above. Estimates of the ratio between ATP and total radioactive nucleotide demonstrated that nearly 90% of the substrate was intact at the end of the incubation period.

Protein concentrations in all of the foregoing procedures was determined by the method of Lowry _et al_. (196).

c. Electrical Microscopic Study of Plasma Membrane

The pellet of the plasma membrane fraction was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 1 hour on ice and rinsed in cold buffer overnight. The pellet was then post-fixed in 1% osmium tetroxide in 0.1 potassium phosphate buffer at pH 7.3 for 1 hour on ice. Dehydration in a graded series of ethanol (70%, 95%, and 100%) solutions on ice was followed by treatment with propylene oxide for 5 minutes twice, and embedded in Epon 812.

Ultra-thin (sliver) sections were cut on a Porter-Blum MT-2 Ultramicrotome with a diamond knife. Sections were stained with uranyl acetate (197) followed by lead citrate (198) and examined in a Philips 201 electron microscope.

2. Renal Cyclic AMP-Dependent Protein Kinase

a. The Standard Assay for Cyclic AMP-dependent Protein Kinase Activity

The reaction mixture (50 or 100 μl) contained 0.05 M TES (2-{[2-hydroxy 1,1-bis (hydroxylmethyl) ethyl] amino }ethane sulfonic acid) buffer (pH 7.0) or 50 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (the specific activity of [γ-^{32}P]ATP ranged from 50-100 cpm/pmol),

1-2 mg/ml histone (Sigma type II calf thymus), no cyclic AMP or 1 μM cyclic AMP, and 50-100 μg of protein. Incubation was carried out at 30°C for 5 min or 10 min. One unit of kinase activity was defined as the amount of enzyme necessary to catalyze the transfer of 1 nmole of ^{32}Pi from [γ-^{32}P]ATP to histone per minute at 30°C. Phosphodiesterase activity was inhibited by adding theophylline to a final concentration of 1 mM. The reaction was stopped by addition of 20 μl 30% trichloroacetic acid. Then 50 μl of aliquot were spotted on a Whatman No. 31ET paper chromatography strip measuring 2 cm x 20 cm. The chromatogram was developed according to the method of Li and Felmly (199).

b. Endogenous Phosphorylation

Phosphorylation of the plasma membrane preparation was assayed by the "standard" procedure outlined above except that NaF was added to a final concentration of 10 mM (to inhibit phosphatase activity) and plasma membrane replaced histone as substrate.

c. Solubilization of Membrane-Bound Protein Kinase

The purified papillary plasma membrane (PPM) from dog kidney was solubilized by adding Triton X-100 to the membrane protein sample to a final concentration of 1%, incubating at 4°C for 2 hours with stirring and then subjecting the incubate to ultracentrifugation at 100,000 xg in the Beckman swinging bucket rotor (SW 27) for 1 hour. The supernatant thus contains the solubilized membrane fraction. In some experiments sodium deoxycholate (DOC) at a final concentration of 1% or ammonium chloride at a final concentration of 1 M, replaced Triton X-100 as the solubilization agent.

d. Purification of Membrane-Bound Protein Kinase

(1) DEAE-Sepharose CL-6B Chromatography of the Solubilized Papillary Plasma Membrane Fraction

Fifty milliliters of solubilized membrane protein (15 mg/ml) was dialyzed for 24 hours against Buffer A (20 mM Tris·HCl, pH 7.0; 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 20 mM KCl), with Triton X-100 at a final concentration of 0.5%. Buffer A with Triton X-100 at this concentration is referred to hereafter as buffer A-T or A-T solution. The ratio of dialysate to dialysand was 1:100; the dialysand was changed twice over the 24-hour period. The dialyzed fraction was subjected to chromatography on a DEAE-Sepharose column (1.6 x 50 cm) pre-eluted with Buffer A-T.

After the sample was applied, the column was washed with 400 ml of the buffer A-T and then eluted with a linear gradient of KCL over a concentration range from 20 mM to 400 mM (with a volume of 600 ml). The fractions were collected with an LKB fraction collector, Model No. 7000, and the conductivity of each fraction was measured with a conductivity meter. Protein concentration was determined by measuring the optical density of the eluent at 280 nm in a Beckman UV spectrophotometer, Model No. 25. The protein kinase activity of every third fraction was assayed and the active fractions were pooled accordingly, each to be concentrated by ultrafiltration using an Amicon membrane (PM-10) to reduce the volume of the pooled fractions to approximately 5 to 10 ml for dialysis against the A-T solution for 24 hours.

(2) Sephadex G-200 Chromatography

The active fractions eluted from the DEAE-Sepharose CL-6B column were applied to a Sephadex G-200 column (1.6 x 75 cm) in which the G-200 beads had been preswelled in A-T solution. The latter column was then eluted with A-T solution and the fractions assayed for protein kinase activity. The active fractions were pooled and concentrated by ultrafiltration through an Amicon membrane (PM-10), to reduce the volume of pooled fractions to approximately 5 to 10 ml and then dialyzed against Buffer A-T.

(3) DEAE-Sephadex A-50 Chromatography

The pooled protein kinase-containing fractions from the Sephadex G-200 column were further chromatographed on a DEAE-Sephadex A-50 column (0.5 x 20 cm) in which the A-50 beads were preswelled in Buffer A-T. After the sample was applied, the column was eluted with a linear salt KCL gradient ranging from 50 mM KCl to 350 mM KCL. The protein kinase activity of each fraction was assayed. The active fractions were pooled and concentrated by Amicon membrane ultrafiltration and then dialyzed against Buffer A-T overnight.

e. Purification of Renal Cortical and Medullary Cytosolic Protein Kinase

(1) Homogenate

The cytosolic fraction of renal tissue was extracted by the following procedure:

2 Kg of canine kidney tissue were cut into small pieces by hand with a pair of scissors and homogenized (weight:volume = 1:3) in 50 mM potassium phosphate buffer (pH 7.0) in a Waring blender for 3 x 10 sec. The homogenate was filtered through 2 layers of cheese

cloth and centrifuged at 10,000 xg for 10 minutes. The supernatant was saved and added to an equal volume of 50 mM potassium phosphate buffer (pH 7.0). The pellet was resuspended in the same buffer, reextracted one more time and recentrifuged at 10,000 xg. The supernatant fluid was saved and recombined with the initial supernatant to serve as a crude extract of the cytosolic fraction.

(2) Ammonium Sulfate Fractionation

The crude extract was precipitated by slow addition of ammonium sulfate with stirring to 50% saturation (0.35 g added per ml). The precipitate was collected by centrifugation at 10,000 xg for 10 min, dissolved in 50 mM Tris·HCl 1 mM, 2-mercaptoethanol, pH 7.0 (T-M Buffer) in a volume amounting to 10% of the original volume of crude extract. The resulting solution was dialyzed against this T-M buffer overnight.

(3) DEAE-Sephadex A-50 Fractionation

The fraction derived from the previous ammonium sulfate precipitation was absorbed in DEAE-Sephadex A-50 gel previously equilibrated in the same T-M buffer. The gel was washed with T-M buffer (using an amount equal to three times the bed volume) and filtered through chromatographic paper (Whatman No. 54). The protein was extracted twice with a 0.3 M potassium phosphate buffer, pH 7.0, each extraction utilizing the same amount of buffer as the bed volume of the gel. The eluent was collected and its protein content precipitated by 70% ammonium sulfate saturation. The precipitate was suspended in a small volume of 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight.

(4) DEAE-Cellulose Chromatography

The enzyme fraction from previous procedure was applied to a DEAE-cellulose (Whatman DE-52) column (2.6 x 45 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0. After applying the sample, potassium phosphate buffer (5 times the bed volume) was passed through the column in order to remove the unabsorbed materials. The enzyme was then eluted with a linear gradient (50 mM to 400 mM) of potassium phosphate, pH 7.0. The fraction containing Type I and Type II protein kinase activities were pooled separately. The enzyme was subjected to ammonium sulfate fractionation as described above.

(5) Alumina Cγ Absorption

The type II protein kinase was further purified by absorption onto Alumina Cγ gel (Bio-Gel). This gel was previously equilibrated with 50 mM potassium phosphate buffer (pH 7.0). After the enzyme was absorbed, the gel was gently stirred for 1 hour and centrifuged at 5,000 xg for 5 min. The supernatant was discarded, the gel was then resuspended in the same volume of the 50 mM potassium phosphate buffer, recentrifuged and the supernatant was again discarded. The gel was then suspended in the same volume of 200 mM potassium phosphate buffer (pH 7.0), stirred for 10 min, centrifuged at 5,000 xg and the extracted supernatant was kept. The gel was extracted two more times using the same procedure. The supernatant fluids (i.e., the extracts of the Alumina Cγ gel) were pooled, concentrated by ultrafiltration through an Amicon (PM-10) membrane, and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol.

(6) Sephacryl S-200 Gel Filtration

The fraction extracted from Alumina Cγ absorption was filtered through a Sephacryl S-200 column (1.6 x 75 cm), which was pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol. The column was eluted with the same buffer. The fractions with protein kinase activity were pooled and concentrated by ultrafiltration with an Amicon (PM-10) membrane.

(7) DEAE-Sepharose CL-6B Chromatography

The fraction derived from the Sephacryl S-200 gel filtration was applied to a DEAE-Sepharose column CL-6B (1.2 cm x 30 cm) pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol. After sample application, the column was washed with 100 ml of 50 mM potassium phosphate-10% glycerol buffer. The protein kinase activity was eluted by a linear gradient of potassium phosphate (50 mM to 400 mM) containing 10% glycerol. The active fractions were pooled, concentrated by ultrafiltration through an Amicon (PM-10) membrane, and dialyzed against 50 mM potassium phosphate (pH 7.0) containing 10% glycerol.

f. Cyclic AMP Binding Assay

The cyclic AMP binding assay was carried out by a modification of the Millipore filter technique of Gilman (200). The binding assay mixture contained 20 mM Tris·HCl, (pH 7.0), 4 mM magnesium acetate, 20 μg/ml of bovine serum albumin, 40 nm of [^{3}H]-cyclic AMP (specific activity approximately 5 x 10^4 dpm per pmol), and the binding protein in a final volume of 100 μl. The assay was started by addition of [^{3}H]-cyclic AMP and the mixture was incubated at 23°C

for 15 min. After incubation, the mixture was diluted with 2 ml of cold 20 mM potassium phosphate (pH 7.0), containing 4 mM magnesium acetate, and filtered through a Millipore filter (HA 0.45 micron pore size), which had been presoaked in the same buffer. The filters were washed four times with 3 ml of cold 20 mM potassium phosphate (pH 7.0) containing 4 mM magnesium acetate.

The filters were dried, transferred to scintillation vials and dissolved in 1 ml of cellosolve. Then 10 ml of a mixture of 3 parts of toluene and 1 part of cellosolve containing 4 g of PPO and 50 mg of POPOP were added to each vial.

g. Autophosphorylation

Autophosphorylation of the purified cyclic AMP-dependent protein kinase (CPK) preparation (see above) was evaluated by two methods. The first method consisted of our standard protein kinase assay (see above) except that no membrane, histone or other substrate was added to serve as phosphate acceptor. In the second method which was carried out according to Erlichman *et al.* (136), the purified cyclic AMP-dependent protein kinase preparation (approximately 50 μg) was added to 50 μl of a solution containing 50 mM potassium phosphate buffer (pH 7.1), 10 mM $MgSO_4$ and 50 μg of bovine serum albumin. The reaction was initiated by addition of 50 μM [γ-^{32}P]ATP (200 cpm/pmol) and continued during incubation 10 min at 37°C. The reaction was stopped by addition of 250 μl of 0.1 M EDTA (pH 5.0). The contents of the tubes were then poured onto a Millipore filter (HA 0.45 micron pore size) which quantitatively retains the enzyme and cyclic AMP binding protein. The filters were washed twice with 10 ml of the 0.1 M EDTA solution, then

dried and assayed for ^{32}P in scintillation fluid containing 4 g of omniflor/liter of toluene.

h. Determination of Molecular Weight of Protein Kinase

The molecular weight of protein kinase was estimated by Sephadex G-200 Gel filtration. The purified protein kinases were filtered through a Sephadex G-200 column (1.6 x 75 cm) previously calibrated. The following proteins were used as standards:

γ-Globulin	(Mr = 167,000)
lactate dehydrogenase	(Mr = 140,000)
alcohol dehydrogenase (horse liver)	(Mr = 80,000)
Bovine serum albumin	(Mr = 67,000)
Myoglobin	(Mr = 17,800)

i. The Preparation of the Regulatory Subunit of Cyclic AMP-Dependent Protein Kinase

The purified holoenzyme of type II cyclic AMP-dependent protein kinase (CPK II) was applied to a column of CM-Sephadex C-50 in the presence of cyclic AMP. Cyclic AMP was added to the enzyme to a concentration of 100 μM in 20 mM potassium phosphate (pH 7.0) buffer. The regulatory subunit was not retained by the column. The eluent was concentrated by ultrafiltration through an Amicon membrane (PM-10). After washing the column with phosphate buffer (20 mM potassium phosphate, pH 7.0), the catalytic subunit was eluted by 2 column volumes of phosphate buffer (300 mM potassium phosphate, pH 7.0).

The membrane-associated protein kinase (MPK II) was eluted with cyclic AMP 100 μM in A- solution (20 mM Tris·HCl, pH 7.0, 1 mM

-mercaptoethanol, 5 mM $MgCl_2$, 20 mM KCl with 5% Triton X-100).

j. G-100 Sephadex Gel Filtration of Regulatory Subunit of Type II Membrane-Associated Protein Kinase (MPK II)

The regulatory subunit of membrane protein kinase MPK II obtained from CM Sephadex C-50 was concentrated by ultrafiltration with an Amicon (PM-10) membrane and dialyzed against the A-T solution for 4 hr. 3.6 mg (1.2 mg/ml) of regulatory subunit combined with 11.2 mg (2.8 mg/ml) of purified protein kinase MPK II was filtered through a G-100 Sephadex column (1.6 x 64 cm) pre-equilibrated with A-T solution. The protein kinase activity and [^{3}H]-cyclic AMP binding activity were determined. The molecular weight of regulatory subunit was calibrated with phosphorylase a (Mr = 94,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000) and myoglobin (Mr = 18,000) as standards.

3. Protein Phosphatase

a. Preparation of ^{32}P Label Phosphoprotein Substrate

[γ-^{32}P]-ATP was purchased from New England Nuclear. Calf thymus histone mixture (Type II) and rabbit skeletal muscle phosphorylase b (2 x crystallized) was purchased from Sigma. Vitamin-free casein, obtained from Nutritional Biochemicals, was dephosphorylated according to Reimann *et al*. (201). Prior to use, cyclic AMP-dependent protein kinase was partially purified from beef heart by a modification of the method of Miyamoto *et al*. (202), involving the elution of the enzyme from a DEAE-cellulose column with a linear gradient (5 to 400 mM) of potassium phosphate buffer (pH 7.0). Histone and/or casein were used as substrate(s). A single peak of cyclic AMP-

dependent protein kinase activity was eluted at about 0.15 M potassium phosphate. The enzymatic activity with either histone or casein as substrate was stimulated approximately 5 to 10-fold by 2 μM cyclic AMP. The protein kinase preparation had a specific activity of about 100 units/mg which represents a 200-fold purification when compared with the activity of the crude extract.

Histone and casein were phosphorylated with $[\gamma^{32}P]$ATP and cyclic AMP-dependent protein kinase was prepared as described. A typical reaction mixture for phosphorylation of histone or casein contained (2- {[2-hydroxy-1.1-bis (hydroxyl-methyl)ethy] amino} ethane sulfonic acid (TES) buffer (pH 7.0), 10 mM $MgCl_2$, 2.6 mg/ml of histone mixture or 10 mg/ml of casein, 2 μM cyclic AMP, 0.5 mg/ml of protein kinase, 0.2 mM $[\gamma^{32}P]$-ATP (250-500 cpm/pmol. Reactions were carried out at 30°C for 3 to 4 hr. After incubation, ^{32}P-labeled protein was isolated and alkali-labile $[^{32}P]$-phosphate content of the phosphorylated protein was analyzed by the method of Meisler and Langan (203). Phosphorylase b was converted to ^{32}P-labeled phosphorylase a by a modification of the method of Torres and Chelala (204). The alkali-labile $[^{32}P]$-phosphate content of a typical preparation of phosphorylated substrates was 30-40 nmol/mg of histone, 2.5-5 nmole/mg of casein and 8-10 nmol/mg of phosphorylase a. The specific radioactivity of ^{32}P-labeled protein used in phosphoprotein phosphatase assays varied from 50-500 cpm/pmol of $[^{32}P]$phosphate.

b. Standard Assay of Protein Phosphatase

Protein phosphatase activity was measured according to Hsiao *et al.* (205) using a standard assay mixture containing 50 mM Tris HCl

(pH 7.4), 1 mM dithiothreitol, 5 mM $MnCl_2$, 0.1 M KCl, and 10 μM ^{32}P-labeled protein; the total incubation volume was 50 μl and the temperature of incubation was 30°C. The reaction was initiated by the addition of the enzyme preparation in an amount which would release less than 15% of ^{32}P-phosphate from the ^{32}P-labeled phosphoprotein by the end of a 10 min incubation period. Enzymatic activity was stopped by addition of 20 μl of 8% trichloroacetic acid to the 50 μl reaction mixture. A 50 μl aliquot of the reaction mixture was then removed and spotted on a 2 x 22 cm Whatman 31 ET chromatographic paper strip, which was transferred to a trough containing 5% trichloroacetic acid for the development of a descending chromatogram at room temperature. The enzymic product, [^{32}P]-phosphate, traveled with the solvent front and was completely separated from the unreacted ^{32}P-labeled proteins which remained at the origin. In less than 20 minutes, the solvent front traveled about 12 cm from origin and the wetted paper strip extending back 4 cm from the solvent front (which contained the [^{32}P]-phosphate) was separated, rinsed in ether and dried under an infrared lamp. The strip was then placed into a vial containing 10 ml of scintillation fluid (10.1 g of P-bis[2-(5-phenyloxazolyl)] benzene and 6 g of 2,5 diphenyloxazole per liter of toluene). The radioactivity was determined in a liquid scintillation counter. This paper chromatographic assay procedure has been compared with the assay procedure described by Meisler and Langan (203), and the results were identical. One unit of phosphohistone phosphatase activity was defined as the amount of enzyme which catalyzed the formation of 1 nmole of [^{32}P]-phosphate/min under conditions outlined above.

c. Preparation of Membrane-bound Protein Phosphatase

(1) Solubilization of Membrane-bound Protein Phosphatase

The membrane-bound phosphatases were solubilized by the following 3 different methods:

a) The membrane fraction was incubated with 1 M NH_4Cl for 1 hr and subjected to ultracentrifugation at 100,000 g for 1 hr. The supernatant was removed and put into cellophane bags for dialysis against buffer A (volume 1:100) overnight with one change of buffer. The dialysate contained solubilized phosphatase activity.

b) The second method involved incubation of the membrane fraction with 1% deoxycholate for 1 hr followed by ultracentrifugation at 100,000 g for 1 hr. The supernatant was removed and put into a cellophane bag for dialysis against Buffer A (volume 1:100) overnight with one change of buffer. The dialysate contained soluble phosphatase activity.

c) The third method was similar to the second method (see above) except that the detergent was replaced by 1% Triton X-100.

(2) DEAE Sepharose CL-6B Chromatography

The dialysate containing solubilized phosphatase activity was applied to a column (1 x 30 cm) of DEAE-Sepharose CL-6B materials previously equilibrated with Buffer A. After the sample was applied to the column, it was rinsed with 200 ml of Buffer A. The column was eluted with 500 ml of a linear salt gradient (50 mM to 400 mM KCl). The major peak and minor peak activities were separated and pooled, concentrated by membrane ultrafiltration

(Amicon, PM-10) to 5-10 ml and dialyzed against Buffer G (20 mM Tris HCl, pH 7.4, 10 mM 2-mercaptoethanol, 100 mM KCl, 5 mM $MgCl_2$, and 10% glycerol).

(3) Sephadex G-100 Chromatography

The concentrated fraction of the major peak of phosphatase activity obtained from the DEAE-Sepharose column was filtered through a Sephadex G-100 column (1.5 x 70 cm) pre-equilibrated with Buffer G (20 mM Tris, pH 7.0; 1 mM 2-mercaptoethanol; 5 mM $MgCl_2$, 20 mM KCl; 10% glycerol). The latter column was eluted with the same buffer. The eluted fractions were pooled and concentrated to between 5 and 10 ml by membrane ultrafiltration (Amicon PM-10) and then dialyzed against Buffer G.

(4) Sephadex G-200 Chromatography

The minor peak of phosphatase activity eluted from the DEAE-Sepharose column was filtered through a Sephadex G-200 column (1.5 x 70 cm), pre-equilibrated with Buffer G and the column was eluted with the same buffer. The active fractions were pooled, concentrated to 5 to 10 ml by membrane ultrafiltration (Amicon PM-10), and then dialyzed against Buffer G (120 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol and 50% glycerol).

(5) DEAE-Sephadex A-50 Chromatography

The major peak of phosphatase activity eluted from Sephadex G-100 was rechromatographed on a column of DEAE-Sephadex A-50 (1 x 30 cm), pre-equilibrated with Buffer G. The column was rinsed with approximately 100 ml of Buffer G to which KCl was added to a final concentration of 50 mM. The enzyme was eluted with a linear gradient of

KCl (400 ml, 100 mM to 300 mM) in Buffer G. The fractions containing phosphatase activity were pooled and concentrated to 5-10 ml by membrane ultrafiltration (Amicon PM-10) and dialyzed against Buffer S (120 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, and 50% glycerol) and stored at -20°C.

All operations were carried out at 4°C. Protein concentration was determined by the method of Lowry et al. (196).

d. Preparation of Cytosol Protein Phosphatase

(1) Crude Extract

The cytosolic fractions of the renal medulla and of the renal cortex were prepared as previously described in the section on isolation of protein kinase except that Buffer A was used instead of the phosphate buffer.

(2) Ammonium Sulfate Fractionation

Solid ammonium sulfate was added to the crude extract to 55% saturation. The resulting precipitate was resuspended in an amount of Buffer A equal to one-tenth of the original volume and then dialyzed against Buffer A overnight.

(3) DEAE-Sephadex A-50 Fractionation

The enzyme in the dialysate obtained in the previous procedure was mixed with DEAE-Sephadex A-50 (Pharmacia) presaturated with Buffer A. The mixture was stirred for an hour and then filtered through Whatman No. 54 filter paper. The resin was washed twice with 2 bed volumes of Buffer A and three times with 2 bed volumes of Buffer A containing 0.05 M KCl. The enzyme adsorbed on DEAE-Sephadex was extracted with 2 volumes of Buffer A containing 0.45 M

KCl, stirred for 1 hr and filtered through Whatman No. 54 filter paper. This extraction process was repeated twice with 1 bed volume of Buffer A containing 0.45 M KCl following which the enzyme in the extract was concentrated by precipitation with addition of solid ammonium sulfate to 70% saturation and dialyzed overnight against Buffer A.

(4) DEAE-Cellulose DE-52 Chromatography

The enzyme solution derived from the procedure described above was applied to a DEAE-cellulose (Whatman DE-52) column (2.6 x 60 cm) previously equilibrated with Buffer A. Then the column was rinsed with 3 bed volumes of Buffer A containing 50 mM KCl, and the enzyme was eluted with a salt gradient (100 mM KCl to 400 mM KCl in Buffer A). The fractions containing enzymatic activity (peak I and peak II) were pooled and concentrated by precipitation with ammonium sulfate to 70% saturation. The precipitate was resuspended in a small volume of Buffer A and dialyzed against Buffer A overnight.

(5) Ethanol Precipitation

One volume of each of the pooled active fractions derived from the procedure described above was mixed with 5 volumes of 95% ethanol at room temperature, and the mixture was centrifuged immediately at 10,000 xg for 10 min. The enzyme in the precipitate was extracted four times with one volume of Buffer A. Each of the ethanol extracts was concentrated by precipitation with $(NH_4)_2SO_4$ to 70% saturation and dialyzed overnight against Buffer A.

(6) DEAE-Sephadex A-50 chromatography

The ethanol treated pooled fraction obtained from the previous procedure were separately chromatographed on DEAE-Sephadex A-50

columns (1.5 x 30 cm) pre-equilibrated with Buffer A containing 0.05 M KCl. After sample application, the column was rinsed with one bed volume of Buffer A-0.05 M KCl. The enzymes were eluted by a salt gradient (0.1 to 0.4 M KCl in Buffer A). Because the enzyme eluted from each column had identical chromatographical profiles, aliquots of the two ethanol-treated fractions were applied to a single DEAE-Sephadex (A-50) column (1.5 x 30 cm) and eluted by a salt gradient (0.1 to 0.4 M KCl in Buffer A), resulting in a single peak of phosphatase activity. Therefore, the two ethanol-treated fractions were pooled and concentrated to a small volume (5 to 10 ml) by ultrafiltration through an Amicon (PM-10) membrane which was then dialyzed overnight against Buffer G (Buffer A + 10% glycerol).

(7) Sephadex G-100 Chromatography

The enzyme fraction obtained from the previous procedure was passed through a Sephadex G-100 column (2 x 65 cm) pre-equilibrated with Buffer G. The column was eluted with the same buffer.

All operations were carried out at 4°C unless otherwise specified.

e. Sucrose Density Gradient Centrifugation

Centrifugation was carried out with an SW 50.1 swinging bucket rotor run at 39,000 rpm for 16 hours at 4°C in a Beckman Spinco Model L2-65B ultracentrifuge. A linear sucrose density gradient (5 ml, 5-20% sucrose in 20 mM Tris·HCl, pH 7.4) was employed. The following marker proteins, bovine serum albumin (2 mg), pig heart malic dehydrogenase (20 μg) and horse heart cytochrome c (0.4 mg), were applied to each gradient in a total volume of 100 μl. After centrifugation, each gradient tube was fractionated and assayed for the marker proteins and for phosphatase activity.

f. Determination of Molecular Weights of Protein Phosphatase

The molecular weight of protein phosphatase was determined by gel filtration. Gel filtration was carried out with Sephadex G-100 and Sephadex G-200. The following proteins were used as internal standards:

γ-globulin (Mr = 167,000)

lactate dehydrogenase (Mr = 140,000)

alcohol dehydrogenase (Mr = 80,000)
(horse liver)

Bovine serum albumin (Mr = 67,000)

Ovalbumin (Mr = 43,000)

Myoglobin (Mr = 17,800)

4. Phosphorylation-Dephosphorylation of Renal Plasma Membrane

a. Preparation of Phosphorylated Plasma Membrane

The ^{32}P-labeled phosphorylated papillary plasma membrane (PPM) was prepared by incubation of 4 mg of membrane protein with 0.5 mM [γ-^{32}P]ATP (approximately 500-1,000 cpm/pmol, 5 mM $MgCl_2$, 50 mM potassium phosphate (pH 7.0), 20 mM NaF, 2 μM cyclic AMP, 0.1 mM theophylline, and 0.5 mg/ml bovine cardiac protein kinase (partially purified by the method of Miyamoto _et al_. (202) in a final volume of 2 ml. The reaction was carried out at 25°C for 1 hr and terminated by addition of 5 ml of 10% trichloroacetic acid followed by centrifugation at 1500 xg for 5 min. The pellet was washed twice by addition of 1 ml of 0.5 M NaOH and 10 μl of 10 mM ATP, and then reprecipitated with 10% trichloroacetic acid after one more washing with water by dilution and centrifugation. The protein was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) at 4°C for 24 hours. Because of the possibility that use of this method leads to

denaturation of the ^{32}P-labeled PPM, an alternative method was utilized. This alternative method involved incubation for 20 min at pH 7 at 25°C. 10 ml 50 mM potassium phosphate buffer was added and centrifuged at 100,000 g for 40 minutes. Dilution with buffer solution and centrifugation were repeated twice and the pellet was dissolved in 50 mM potassium phosphate buffer (pH 7.0) to yield native ^{32}P-labeled PPM.

b. Determination of Renal Plasma Membrane Phosphorylation

Assay of the degree of phosphorylation of renal plasma membrane was carried out following the standard assay described above, except that the incubation time was prolonged to 20 min, and the membrane-associated phosphatase activity was inhibited by the addition of either 10 mM NaF, 2 mM pyrophosphate or 5 mM Zn^{++}. The plasma membrane was phosphorylated either by endogenous protein kinase, or the purified soluble protein kinase or the catelytic subunit of protein kinase.

c. Polyacrylamide Gel Electrophoresis

Disc electrophoresis on 7% polyacrylamide gel was carried out by the procedure of Davis and Ornstein (206). After the electrophoresis, the gel was either stained with Coomassie brilliant blue or transversely sliced into 1 mm sections and assayed for the following:

1. Cyclic AMP dependent protein kinase activity
2. Protein phosphatase activity
3. [^{3}H] cyclic AMP binding
4. Autophosphorylation

The sliced sections were placed into the test tubes containing standard assay mixture for each enzyme. The assays were carried out

by "standard assay methods" (see above), and incubated at 30°C for 20 min. Autophosphorylation was detected by putting the sliced sections in toluene scintillation fluid (4.0 g PPO and 0.2 g POPOP) and counting in a Packard liquid scintillation counter.

Sodium dodecyl sulfate gel electrophoresis was performed according to the procedure of Laemmli (207) employing a 10% polyacrylamide gel. Bovine serum albumin, ovalbumin, cytochrome c and myoglobin were used as internal standards. Autophosphorylation was detected also by placing the sliced sections in toluene scintillation fluid (4.0 g PPO and 0.2 g POPOP) and counting in a Packard liquid scintillation counter.

d. Cyclic AMP-Dependent Dephosphorylation

Cyclic AMP-dependent dephosphorylation was determined after (1) incubating the membranes with [γ-^{32}P]ATP for 3 minutes at 30°C in the presence of Zn^{++} to inhibit phosphatase activity (the assay mixture contained 100-500 μg of membrane protein, 50 mM MES (pH 6.2), 1 or 5 mM $ZnCl_2$, 10 μM ATP (500 cpm/pmol), total volume 100 μl and (2) then adding 30 μl of a solution containing 195 mM EDTA, 0.1 mM ATP and 195 μM cyclic AMP. The latter reaction mixture was incubated for 10 minutes and the reaction was stopped by adding 1 ml of ice cold 40% TCA. The assay tubes were centrifuged in a Brinkmann 3200 table centrifuge for 1 minute. The supernatants were discarded. Then the same volume of H_2O was added and the mixture was recentrifuged. The H_2O was discarded and the pellet suspended in 50 μl of SDS solubilizing solution (2% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol, 20 mM sodium phosphate, pH 7.0). The tubes were placed in boiling water until the pellet was solubilized; then 80 μl of solution B (containing 0.1 mM EDTA, 1% 2-mercaptoethanol,

20 mM sodium phosphate, pH 7.0, 40% sucrose, and 0.005% bromphenol blue) were added. Thereafter, 50 μl of the final solution were subjected to disc and slab gel electrophoresis.

e. Slab Gel Electrophoresis and Autoradiography

The slab gel electrophoresis was carried out for 8-10 hr at 30-50 mA on a vertical plate gel of 7% polyacrylamide in the presence of 1% SDS. The gel was run by the method described by Fairbanks *et al.* (208). The gel dimensions were 11.5 cm x 12.8 cm x 1 mm, and the apparatus used was that described by Reid and Bieleski (209). The gel was stained for protein with 0.0025% Coomassie Blue in 10% isopropyl alcohol, 10% acetic acid and destained with several changes of 10% acetic acid. The stained gel was dried on Whatman No. 50 filter paper under vacuum and heat, and placed in close contact with Kodak Royal X-Omat film. The film was exposed for 2-8 days depending upon the level of radioactivity in the dried gel that was to be developed.

III. RESULTS

1. Purification of Plasma Membrane from the Inner Medulla of Canine Kidney (Papillary Plasma Membrane)

The purification of renal papillary plasma membrane (PPM) was monitored by measurement of marker enzyme activities (Table 1) and by electron microscopy (Fig. 1).

5'-Nucleotidase, the widely accepted plasma membrane enzyme marker (210) was increased four-fold in specific activity in comparison to homogenate by the purification procedure. Glucose-6-phosphatase was used as a supplementary enzyme marker for the microsomal fraction (endoplasmic reticulum). The activity of glucose-6-phosphatase relative to 5'-nucleotidase can indicate contamination of PPM by endoplasmic reticulum. The purification procedure resulted in a slight decrease in the glucose-6-phosphatase activity (Table 2). The activity of Na^+-K^+ ATPase, a basic enzyme marker for plasma membrane (211), was increased five-fold, and HCO_3-ATPase, a particular marker for PPM (99), three-fold in comparison to the homogenate.

Contamination of PPM by mitochondrial membrane is indicated by succinate dehydrogenase activity. The specific activity of this enzyme in the mitochondrial fraction was increased ten-fold compared to the homogenate, but showed almost no increase in the PPM fraction.

The adenylate cyclase activity of PPM in the presence of 10 mM NaF was 80% greater than that of the NaF-treated homogenate, whereas the specific activity of the PPM enzyme was increased by 3.5-fold in the presence of 10 mM NaF in comparison to the basal level (table 3).

Table 1: Specific Activities of Enzyme Markers for Subcellular Fractions from Cells of Canine Renal Medulla.*

	Homogenate fraction	Mitochondria fraction	Cytosol fraction	PPM fraction
5' Nucleotidase	0.36	0.33	--	1.5
Glucose-6-Phosphatase	2.7	5.6	--	2.1
Na^+-K^+ ATPase	2.3	1.5	0.3	12.1
HCO_3^--ATPase	1.5	1.15	0.6	4.9
Succinate Dehydrogenase (U/mg)	0.11	1.1	--	0.14

*Enzymatic activity for 5' nucleotidase, glucose-6-phosphatase, Na^+-K^+ ATPase and HCO_3^--ATPase is defined as follows: one unit equals one micromole orthophosphate liberated per hour per mg of protein. The activity for succinate dehydrogenase is based on an arbitrary unit.

Table 2: <u>Relative Specific Activities of Enzyme Markers of Subcellular Fractions of Canine Renal Medulla</u>.*

	Homogenate	Mitochondria	Cytosol	PPM
5' Nucleotidase	100	92	0	416
Glucose-6-Phosphatase	100	208	0	77
Na^{+}-K^{+} ATPase	100	65	13	520
HCO_3^{-}-ATPase	100	76	40	320
Succinate Dehydrogenase	100	990	--	127

*This table is based on the data in Table 1 with an assignment of a value of 100 to the homogenate.

Table 3: The Activity of Adenylate Cyclase.*

	Homogenate	PPM
Basal	16.4	23.6
Fluoride (10 mM)	46.9	84.4

*Adenylate cyclase activity of PPM and homogenate is shown in the table. The concentration of NaF is 10 mM. The unit of activity is pmol cyclic AMP generated per min per mg protein.

A study of the membrane preparation by electron microscopy revealed vesicular membrane components, which can be seen in Fig. 1. The vesicles occasionally enclosed electron-dense material. A small number of contaminating intact and disrupted mitochondria could be observed.

2. Renal Protein Kinases

a. Subcellular Distribution of Cyclic AMP-Dependent Protein Kinases from the Inner Medulla of Canine Kidney

The subcellular distribution of protein kinase activity was determined with histone as the substrate (Table 4). The cytosol fraction has a specific activity that is approximately 50% higher than that of the papillary plasma membrane (PPM) fraction and a 13-fold greater total activity due to the greater amount of protein in the former fraction. Thus, the majority of protein kinase activity is located in the cytosol fraction, a finding which suggests that protein kinase in the cytosol may play a role in the regulation of cyclic AMP-dependent phosphorylation of plasma membrane as well as cytoplasmic components.

b. Solubilization of Protein Kinases from Renal Plasma Membrane

The membrane protein was solubilized by adding the detergents Triton X-100 and sodium deoxycholate (DOC) and the salt NH_4Cl.
The concentration of detergent and salt were as follows:
1% Triton X-100, 1% sodium deoxycholate (DOC), and 1 N NH_4Cl. All solubilization procedures were carried out at 4°C for 2 hours. From the data shown in Table 5, it can be seen that 1% Triton X-100 was

Table 4: Renal Medulla Subcellular Distribution of Cyclic AMP-Dependent Protein Kinase Activity.*

	Specific activity (mU/mg)**	% of Total activity	% of Total amount of protein
Homogenate	241	100%	100%
Debris	148	28%	54%
Mitochondria	60	2%	8%
Cytosol	341	65%	31.9%
PPM	228	5%	5.5%

*The assay was carried out by the standard kinase assay method described in the Experimental Procedure section. Histone was used as substrate. The assay mixture was incubated for 10 min at 30°C.

**One unit (U) of protein kinase activity is defined as that amount of enzyme which catalyzes the transfer of 1 nmol of phosphate from [γ-^{32}P]ATP to substrate per min under the standard assay condition.

the most effective solubilizing agent; 85% of the protein kinase activity was solubilized. DOC at a final concentration of 1% brought 65% of the total kinase activity into solution, whereas 1 N NH_4Cl affected very little solubilization of the membrane-bound protein kinase (Table 5). These results suggest that at least some moiety of renal medullary protein kinase is membrane-bound because it cannot be solubilized without detergent. Before solubilization, the phosphorylation of histone by membrane-bound protein kinase was 276 mU/mg in the presence of 1μM cyclic AMP and 135 mU/mg in the absence of cyclic AMP. After solubilization with Triton X-100, these activities were 385 mU/mg and 182 mU/mg, respectively, indicating that the solubilization increased the specific activity of the cyclic AMP-dependent protein kinase. The solubilization also enhanced the cyclic AMP-dependent endogenous phosphorylation of plasma membrane (Table 6).

c. Separation of Membrane-Bound Protein Kinases

(1) Elution Profile

As shown in Fig. 2, two large peaks of membrane-bound protein kinase activity were separated by ion-exchange chromatography on a DEAE-Sepharose CL-6B column. The first (minor) peak eluted at a KCl concentration between 80 and 100 mM; the second (major) peak was eluted at a KCl concentration between 170 and 190 mM. A third peak (shoulder) was eluted at a KCl concentration between 230 and 250 mM. Peak III was not observed consistently.

Peak I is identified as MPK I (membrane-bound protein kinase I), and peak II, as MPK II (membrane-bound protein kinase II). MPK II has

Table 5: The Solubilization of Membrane-Bound Protein Kinase.*

Membrane Solubilization Method	Specific Activity (mU/mg) -cAMP	+cAMP 1 μM	Relative Activity**
1% Triton X-100	182	385	85%
1% DOC	161	315	65%
1N NH_4Cl	--	--	--
Untreated Membrane	135	276	--

*The assay was carried out by the standard kinase assay method described in the Experimental Procedure section. The assay mixture was incubated for 10 min at 30°C. Enzymatic units are defined in a footnote to Table 4.

**The relative activity is the specific activity of the solubilized enzyme relative to that of the unsolubilized enzyme.

Table 6: The Activity of Membrane-Bound Protein Kinase.*

Specific Activity (mU/mg)

	Endogeneous Phosphorylation		Histone as Substrate	
	Unsolubilized Membrane	Solubilized Membrane	Unsolubilized Membrane	Solubilized Membrane
−cAMP	3.3	7.5	135	182
+cAMP 1 μM	5.1	20.1	276	385

*The assay was carried out by the standard kinase assay method described in the Experimental Procedure section. The endogeneous phosphorylation was assayed without addition of histone as substrate.

more than 90% of the total membrane protein kinase activity. Accordingly, MPK I possesses less than 10% of the total membrane-bound protein kinase activity.

(2) Molecular Weights of Membrane-Bound Protein Kinases MPK I and MPK II

The molecular weight of the protein kinases were determined by Sephadex G-200 gel filtration. As shown in Fig. 3, the approximate molecular weight for MPK I is 210,000 daltons and for MPK II is 110,000 daltons. These molecular weight estimations are considered to be only approximations, because the presence of Triton X-100 in the elution buffer could influence differentially the elution of the enzymes and the standard molecular weight markers.

(3) Effects of Cyclic AMP on Membrane-Bound Protein Kinases

When histone is used as the substrate for membrane-bound protein kinase with an optimal cyclic AMP concentration, 5 μM, the increase of phosphorylation compared to basal activity for MPK I is 3-4 fold and for MPK II is 4-5 fold. On the other hand, when unsolubilized membrane is used as substrate, the ability of MPK I for phosphorylation is relatively poor, and the presence of 1 μM cyclic AMP results in only a 50% increase in the specific activity (Table 7). Similarly, MPK II also exhibits low specific enzymatic activity, and the cyclic AMP-induced increment is less than 100% of the basal activity. When membranes solubilized by Triton X-100 are used as substrate, however the specific activity of MPK I increased 150% and that of MPK II increased 200% in the presence of 1 μM cyclic AMP.

Table 7: Phosphorylation of Renal Medullary Plasma Preparation by Membrane-Bound Protein Kinases MPK I and MPK II.*

		Specific Activity (mU/mg)	
		- cyclic AMP	+ cyclic AMP 1 μM
MPK I	Unsolubilized membrane	5.4	8.6
	Solubilized membrane	10.6	27.8
MPK II	Unsolubilized membrane	3.6	8.6
	Solubilized membrane	10.6	16.2
Endogeneous Phosphorylation	Unsolubilized membrane	3.3	5.1
	Solubilized membrane	7.5	12.8

*The assay was carried out in accordance with the standard kinase assay method described in the Experimental Procedure section, except that the membrane replaced histone as substrate, and that 20 mM NaF was added to the assay mixture to inhibit the protein phosphatase activity. The assay mixture was incubated for 10 min at 30°C.

d. Inhibition of Membrane-Bound Protein Kinases MPK I and MPK II by Heat-Stable Inhibitor

When heat-stable inhibitor of protein kinase (extracted from bovine cardiac muscle and purchased from Sigma Chemical Co.) was added to a final concentration of 0.5-1.0 mg/ml to the assay mixture (histone as substrate), the activities of MPK I and MPK II decreased. As the concentration of inhibitor was gradually increased to saturation, the degree of inhibition of MPK I was 75% whereas that of MPK II was 60% (Table 8). When these protein kinases were still associated with the membrane, however, i.e., they were not solubilized, the maximum ihibition affected by the inhibitor was only 40%. This result indicates that these protein kinases are less susceptible to inhibitor when they are bound to the membrane.

e. Binding of [^{3}H]-Cyclic AMP by Proteins Solubilized from Papillary Plasma Membrane

Binding of [^{3}H]-cyclic AMP to membrane fractions eluted from DEAE-Sepharose CL-6B columns showed a quantitatively identical elution pattern to that of the intrinsic cyclic AMP-dependent protein kinase activities corresponding to MPK I and MPK II (Fig. 4). In addition, there was a third peak of binding activity eluted at a KCl concentration of between 0.3 and 0.4 M. Although this third peak binds [^{3}H]-cyclic AMP, it does not manifest protein kinase activity either in the presence or absence of cyclic AMP. The nature of this particular binding protein is unknown.

f. Autophosphorylation of Membrane-Bound Protein Kinases

After elution of renal medullary plasma membranes solubilized by Triton X-100 from a DEAE-Sepharose CL-6B column and determination of

Table 8: The Inhibition of Membrane-Bound Protein Kinases MPK I and MPK II by Heat Stable Inhibitor.*

	Specific Activity (U/mg)		
	MPK I	MPK II	Membrane
-cAMP	1.41	2.81	0.18
-cAMP + inhibitor (2 mg/ml)	0.7	1.12	0.11
+ cAMP	4.32	10.2	0.38
+ cAMP + inhibitor (2 mg/ml)	1.12	4.12	0.15

*The heat stable inhibitor of protein kinase from bovine heart was purchased from Sigma Chemical Co. The solid inhibitor was diluted by buffer A and added to the assay mixture to the final concentration of 2 mg/ml. The assay was carried out by the standard kinase assay method described in the Experimental Procedure section, either in the presence or absence of inhibitor.

the autophosphorylation by the method of Erlichman *et al.* (136), it was found that the fraction that underwent autophosphorylation corresponded exactly to the fractions of MPK II (Fig. 5). When MPK I and MPK II fractions from several different experiments were separately pooled, concentrated and assayed both for cyclic AMP-dependent protein kinase activity and the property of autophosphorylation, both peaks showed comparably high levels of specific activity for this kinase activity (when histone was used as substrate), but only the MPK II fraction demonstrated a high degree of autophosphorylation (the MPK I fraction demonstrated slight or nil autophosphorylation). An identical result was obtained when membranes solubilized by Triton X-100 were eluted from a column of Sephacryl S-200 (Fig. 6) and tested for cyclic AMP-dependent protein kinase activity and autophosphorylation. The autophosphorylation of the MPK II fraction might be the result of a non-cyclic AMP-dependent phosphorylation of unknown substrates eluted at the same region as MPK II. The following findings militate against the latter possibility and favor the conclusion that MPK II is indeed autophosphorylated:

1) SDS-polyacrylamide gel electrophoresis of MPK II showed that the site of autophosphorylation of this peak is consistently located at the Mr = 50,000 dalton region of molecular weight (see section on membrane phosphorylation). As noted above, MPK I shows little or no autophosphorylation.
2) These above results are similar to the finding of Erlichman *et al.* (136) in their studies of the autophosphorylation

of beef heart Type II protein kinase and Uno, et al. (139) in their studies of membrane-bound protein kinase from synaptic membranes.

g. Purification of Membrane-Bound Protein Kinase MPK II

The major membrane-bound protein kinase MPK II was purified approximately 137-fold relative to unsolubilized membranes (Table 9) by the following steps:

1) Triton X-100 solubilization of papillary plasma membrane
2) DEAE-Sepharose CL-6B ion-exchange chromatography
3) Sephadex G-200 gel filtration
4) DEAE-Sephadex A-50 ion-exchange chromatography

h. Stability of Membrane-Bound Protein Kinases

On storage at -25°C, the major membrane-bound protein kinase, MPK II, showed no reduction in enzymatic activity after 3 months, whereas the minor membrane-bound kinase, MPK I, lost all enzymatic activity within 3 weeks.

On storage at 4°C, MPK II and MPK I lost all enzymatic activity within 30 days and 10 days, respectively.

When the concentration of Triton X-100 was reduced to 0.1%, MPK II activity could still be eluted from the DEAE-Sepharose CL-6B column, but little or no MPK I activity could be eluted. The MPK II eluted by this detergent proved to be rather unstable, however, for its enzymatic activity disappeared within a period of 10 days. When Triton X-100 was totally eliminated from the elution buffer, no MPK II or MPK I activities could be eluted from the column. This requirement for Triton X-100 indicates that the protein kinases,

Table 9: Purification of Membrane-Bound Protein Kinase MPK II.*

	Protein (mg)	Specific activity (U/mg)	Total activity (U)
Membrane	350	0.36	126
Triton X-100 solubilization	176	0.72	127
DEAE-Sepharose CL-6B Ion Exchange	29.9	3.80	113
Sephadex G-200 Gel filtration	11.3	8.90	110
DEAE-Sephadex A-50 Ion Exchange	1.9	37.2	70

*The assay of the purified fraction was carried out by the standard kinase assay method, and histone was used as substrate. One unit (U) of protein kinase activity is defined as the amount of enzyme necessary to catalyze the transfer of 1 nmol of orthophosphate from $[\gamma-^{32}P]$ATP to histone per minute under the standard assay condition.

MPK I and MPK II are tightly bound to the plasma membrane. This binding is probably an intrinsic association between enzyme and membrane, because solubilization by the detergent increases endogenous cyclic AMP-dependent protein kinase activity.

i. Separation of Cytosol Protein Kinases of the Renal Cortex and Medulla

Two cyclic AMP-dependent protein kinases were detected in the elution profile of the cytosol fraction from renal medulla by DEAE-cellulose chromatography (Fig. 7). The first protein kinase (CPK I) was eluted at a KCl concentration between 60 and 80 mM, and the second one (CPK II), at one between 150 and 180 mM. CPK II had more than 90% of the total cytosol kinase activity. Elution of the cytosol fraction of renal cortex on the same DEAE-cellulose column resulted in an identical profile (Fig. 8). When the medullary and cortical cytosol fractions were combined, the elution profile was identical to that obtained on chromatography of each of the separate fractions. These results indicate that in the canine kidney, the cytosol protein kinases have similar characteristics and distributions in the cortex and in the medulla. These results also show that the major cyclic AMP-dependent cytosol protein kinase from renal tissue (CPK II) is a type II kinase (133) and accounts for more than 85% of the total kinase activity.

j. Purification of Cytosol Protein Kinase CPK II

Type II cytosol protein kinase (CPK II) from renal tissue was purified (Table 10) by the following steps:

1) Homogenization

2) Ammonium sulfate precipitation

3) DEAE Sephadex A-50 batch

4) DEAE-cellulose DE-52 ion-exchange

5) Alumina C_γ absorption

6) Sephacryl S-200 gel filtration

7) DEAE-Sepharose CL-6B ion-exchange

The purification resulted in over a 400-fold increase in specific activity relative to the homogenate, and the overall recovery was 89% (Table 10). The binding of [^{3}H]-cyclic AMP showed an approximately 500-fold increase in specific activity after the final stage of purification.

k. Substrate Differentiation

Table 11 shows the activities of the protein kinase derived from the renal cytosol crude extract and tested with three different substrates--namely, histone, protamine, and casein. It can be seen that histone is the best substrate for renal cytosol protein kinase and that the medullary fraction had 45% more activity than the cortical fraction. The K_m values for activation by cyclic AMP when histone was the substrate was found to be 66 nM and 72 nM for crude extracts of cortex and medulla, respectively.

l. Binding of [^{3}H]-Cyclic AMP by Autophosphorylation of Cytosol Protein Kinases

The binding of [^{3}H]-cyclic AMP to the cytosol protein kinases CPK I and CPK II is increased in proportion to the degree of purifi-

Table 10: Purification of Cytosol Protein Kinase CPK II.*

	Protein (mg)	Protein Kinase (U/mg)	[^{3}H]cyclic AMP binding (mU/mg)	Total Units (U)
Homogenate	31,800	0.48	0.51	15,292
Ammonium Sulfate	10,832	1.14	1.26	12,149
DEAE-Sephadex A-50	3,448	2.47	2.63	8,491
DEAE-Cellulose DE-52	440.8	10.6	12.1	4,672
Alumina C_{γ}	90.7	34.3	37.9	3,245
Sephacryl S-200	21.4	108	121	2,312
DEAE-Sepharose CL-6B	5	203	247	1,274

*One unit (U) of protein kinase activity is defined as the amount of enzyme necessary to catalyze the transfer of 1 nmol of orthophosphate from [γ-^{32}P]ATP to histone per minute. One unit of cyclic AMP binding is defined as binding of 1 nmol of cyclic AMP under the standard assay condition.

Table 11: Activity of Renal Cytosol Cyclic AMP-Dependent Protein Kinase with Different Substrates.*

	Specific Activity (mU/mg)					
	Histone		Protamine		Casein	
	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP
Renal Cortex	64.1	286	26.2	63.1	9.6	24.5
Renal Medulla	88.1	396	33.1	74.2	12.3	27.8

*The assay was carried out by the standard kinase assay method described in the Experimental Procedure section, except that different substrates were used at the following concentrations: histone, 1.2 mg/ml; protamine, 1.1 mg/ml; casein, 1.4 mg/ml. The concentration of cyclic AMP in the assay was 1 μM.

cation. In the cytosol fractions eluted from the DEAE-cellulose DE-52 column only CPK II appeared to be autophosphorylated. It is possible that the results on autophosphorylation shown in Fig. 9 include an artifact--namely, the phosphorylation of a protein other than CPK II that is eluted along with the purified CPK II fraction. SDS polyacrylamide gel electrophoresis of the purified CPK II fraction revealed a single phosphorylated band characterized as a protein with a molecular weight of 50,000 daltons which is identical to that of the regulatory subunit of CPK II.

m. Molecular Weight of Renal Cytosol Protein Kinase CPK II

The molecular weight of renal cytosol protein kinase CPK II was determined by gel filtration on a Sephadex G-200 column, as shown in Fig. 10. The molecular weight of CPK II is approximately 180,000 daltons.

n. Characteristics of Canine Renal Protein Kinases

(1) Effect of the Concentration of Substrate on Protein Kinase Activity

When histone was used as a substrate for purified membrane-bound protein kinase MPK II and purified cytosol protein kinase CPK II, both enzymes were found to have maximal activity at a substrate concentration of approximately 3 mg/ml (Fig. 11). The K_m for both enzymes was found to be 0.4 mg/ml.

(2) Effects of Divalent Cations on Protein Kinase Activity

a) Mg^{++}

In general, the enzymatic activity of cyclic AMP-dependent protein kinase requires the presence of Mg^{++}-ATP complex (108). The optimal range of Mg^{++} concentration for MPK II and cytosol CPK II is 5-20 mM

(Fig. 12). When Mg^{++} concentration was increased to 50 mM, the protein kinase activity was reduced to 60% of the maximal activity.

b) Mn^{++}

Under the standard assay conditions with 5 mM Mg^{++}, Mn^{++} did not increase cyclic AMP-dependent protein kinase activity. When Mg^{++} was totally replaced by Mn^{++} at a concentration of 5 mM, however, the activity of the enzyme was reduced to 15% of the activity noted in the presence of 5 mM Mg^{++}. Mn^{++} at a concentration of 10 mM caused a 60% inhibition of MPK II, CPK II, and unsolubilized membrane-bound protein kinase. When the Mn^{++} concentration was increased to 50 mM, the protein kinase activity was reduced to 20-25% of the maximal activity (Fig. 13).

c) Ca^{++}

Ca^{++} inhibited the activities of all cyclic AMP-dependent protein kinases at a concentration of 5 mM. The activities of MPK II, CPK II, and unsolubilized membrane-bound protein kinase were reduced to 50% of the maximal activity at this Ca^{++} concentration, and to 30% at a Ca^{++} concentration of 10 mM (Fig. 14).

d) Zn^{++}

Zn^{++} inhibited the activities of protein kinase (Fig. 15) at a concentration of 5 mM. Zn^{++} caused a 55% inhibition of MPK II, CPK II and unsolubilized membrane-bound protein kinase activities. At a concentration of 10 mM, the degree of inhibition was increased to 65%. With a further increase in the Zn^{++} concentration to 50 mM, there was no further increase in the inhibition of the enzyme.

(3) Effects of Monovalent Cations on Protein Kinase Activity

a) Na^+

Na^+ at a concentration of 0.1 M caused an approximately 15% inhibition of MPK II activity, a 10% inhibition of CPK II activity, and a 30% inhibition of unsolubilized membrane-bound protein kinase activity. At a concentration of 0.5 M Na^+ caused a 75% inhibition of unsolubilized membrane-bound protein kinase activity, a 40% inhibition of MPK II activity and a 30% inhibition of CPK II activity (Fig. 16A).

b) K^+

K^+ inhibited cyclic AMP-dependent protein kinase activity only at a high concentration. At 0.1 M, K^+ caused a 20% inhibition of MPK II activity, a 10% inhibition of CPK II activity and a 35% inhibition of the activity of unsolubilized membrane-bound protein kinase activity. At a concentration of 0.5 M, K^+ caused a 25% inhibition of CPK II activity, a 40% inhibition of MPK II activity and a 65% inhibition of unsolubilized membrane-bound protein kinase activity (Fig. 16B).

(4) K_m Values Related to Protein Kinase Activity

The K_m values for cyclic AMP, ATP and Mg^{++} (with histone as substrate) for renal protein kinases CPK I, CPK II and MPK II are shown in Table 12. CPK II has a higher K_m value for cyclic AMP (50 nM) than does CPK I (9 nM). CPK II also has a higher K_m value for Mg^{++} (2.0 mM) than does CPK I (1.0 mM). The K_m value for ATP for both cytosol protein kinases is the same, namely, 16 μM. Membrane-bound protein kinase MPK II has higher K_m value for ATP, Mg^{++}, and cyclic AMP than do the cytosol kinases (Table 12).

Table 12: K_m Value for Renal Cytosol Protein Kinases CPK I, CPK II and Membrane-Bound Protein Kinase MPK II.*

	CPK I	CPK II	MPK II
Value			
Cyclic AMP	0.9 nM	5 nM	12 nM
ATP	16 μM	16 μM	60 μM
Mg^{++}	1.0 mM	2.0 mM	2.5 mM

*The assay was carried out by the standard assay method described in the Experimental Procedure section using histone as substrate.

(5) The pH Optima for Protein Kinases

As shown in Fig. 17, the pH optima for renal cytosol cyclic AMP-dependent protein kinase activity are 6.5-7.0 for CPK I and 7.0-7.5 for CPK II. The pH optimum for type II membrane-bound cyclic AMP-dependent protein kinase (MPK II) is also in the range of 7.0-7.5.

(6) Effect of Cyclic AMP and Cyclic GMP on Protein Kinase Activity

Stimulation of both protein kinases CPK II and MPK II was maximal at a cyclic AMP concentration of 1 μM. Cyclic GMP evoked maximal stimulation of CPK II at a concentration of 10 μM, but the degree of maximal stimulation was far less than observed with cyclic AMP. Cyclic GMP had no effect at a concentration of 0.1 μM (Fig. 18).

3. Renal Protein Phosphatases

a. Subcellular Distribution of Protein Phosphatases from the Inner Medulla of Canine Kidney

The subcellular distribution of protein phosphatase activity was determined with both [^{32}P]-histone and [^{32}P]-casein as substrates (Table 14). With both of these substrates, the cytosol fraction has a 2-fold greater specific activity than the papillary plasma membrane (PPM) fraction and a 13-fold greater total activity due to the greater amount of protein in the former fraction. The table shows that more than 70% of the total activity of protein phosphatase is distributed in the cytosol fraction, a finding which suggests that phosphatase in the cytosol may play a role in the regulation of dephosphorylation of plasma membrane as well as cytoplasmic components.

Table 13: The Comparison of Renal Membrane-Bound Protein Kinase MPK II and Renal Cytosol Protein Kinase CPK II

Nature of enzyme	cytosol fraction major protein kinase	membrane-bound major protein kinase
Type of classification	similar to beef heart type II protein kinase	membrane-tight-bound protein kinase
DEAE chromatography salt elution	0.15–0.18 M	0.17–0.19 M
Molecular weight (Mr)	Mr = 180,000	Mr = 100,000
$[H^3]$-cyclic AMP	binding to $[H^3]$-cyclic AMP	binding to $[H^3]$-cyclic AMP
Autophosphorylation	autophosphorylated the regulatory subunit	autophosphorylated the regulatory subunit
K_m for cyclic AMP	0.9 nM	12 nM
K_m for ATP	16 μM	60 μM

Table 14: Subcellular Distribution of Protein Phosphatase of Renal Medulla.*

	$[^{32}P]$-Histone Specific Activity (mU/mg)	Total Activity	$[^{32}P]$-Casein Specific Activity (mU/mg)	Total Activity
Homogenate	764	100%	280	100%
Debris	405	21%	120	20%
Mitochondria	474	33%	49	0.7%
Cytosol	1717	70.6%	602	74.6%
PPM	560	5.1%	210	4.7%

*The assay was carried out by the standard protein phosphatase assay method described in the Experimental Procedure section. $[^{32}P]$-casein and $[^{32}P]$-histone were used as substrates. One unit (U) of protein phosphatase activity is defined as that amount of enzyme which catalyzes the formation of 1 nmole of orthophosphate per min under the standard assay condition.

b. Solubilization of Protein Phosphatases from Renal Plasma Membrane

Twenty-five percent of the total membrane-bound protein phosphatase from renal medulla can be solubilized by incubating the PPM with 1 M KCl for 1 hour at 4°C. The remaining activity can be solubilized only by the addition of NH_4Cl or detergent. When the membrane preparation was incubated with 1 N NH_4Cl for 1 hour at 4°C, 50% of the total membrane-bound phosphatase activity was solubilized. With 1% sodium deoxycholate, approximately 80% of this activity was solubilized, and with 1% Triton X-100, 92% of this activity was solubilized. When stored at 4°C either in the absence or presence of detergent (0.5% Triton X-100), the solubilized phosphatase activity maintained full activity and proved to be stable for 4 months. When completely solubilized, the phosphatase activity of the membrane fraction is increased by more than 4-fold relative to the activity of the unsolubilized fraction (Table 15).

c. Separation of Membrane-Bound Protein Phosphatase

As shown in Fig. 19, two large peaks of membrane-bound protein phosphatase activity could be resolved by ion-exchange chromatography on a DEAE-Sepharose CL-6B column. Peak I and peak II were eluted at KCl concentrations of 180 mM and 250 mM and associated with phosphatase activities designated Ma and Mb, respectively. Whether [^{32}P]-histone or [^{32}P]-casein was used as substrate, the major peak Ma contained approximately 80% of the total enzymatic activity, and the minor peak Mb, approximately 20%. When fractions from peaks Ma and Mb were assayed in the presence of 5 mM $MnCl_2$, they were both stimulated slightly.

Table 15: Solubilization of Protein Phosphatase from Membrane.*

	Specific Activity (U/mg)	Relative Activity**
Unsolubilized Membrane-Bound Phosphatase Activity	0.75	--
+ 1 M KCl	4.21	25%
+ 1 N NH_4Cl	3.71	50%
+ 1% Sodium deoxycholate	4.31	80%
+ 1% Triton X-100	4.12	92%

*The assay was carried out by the standard protein phosphatase assay described in the Experimental Procedure section. [^{32}P]-histone was used as substrate.

**Relative activity is the specific activity of the solubilized enzyme relative to that of the unsolubilized enzyme.

d. Purification of Membrane-Bound Protein Phosphatase Ma

The major membrane-bound protein phosphatase Ma was purified approximately 75-fold relative to unsolubilized membranes (Table 16) by the following steps:

1) Triton X-100 solubilization of PPM
2) DEAE-Sepharose CL-6B ion-exchange chromatography
3) Sephadex G-100 gel filtration
4) DEAE-Sephadex A-50 ion-exchange chromatography

The purification procedure was carried out in the absence of detergent as well as in the presence of 0.5% Triton X-100 in the elution buffer. It was noted that the presence of detergent affected the stability of the enzyme during storage at -25°C; the enzymatic activity was maintained for several months longer when the elution was carried out without detergent.

e. Separation of Cytosol Protein Phosphatases of the Renal Medulla

Two protein phosphatases were detected in the elution profile of the 55% ammonium sulfate fraction from renal medulla by DEAE-cellulose chromatography. The major peak (Pa) and the minor peak (Pb) were eluted at KCl concentrations of 175 mM and 240 mM, respectively. Phosphatase Pa had 75% and phosphatase Pb had 25% of the total phosphatase activity when either [^{32}P]-histone or [^{32}P]-casein was used as the substrate. The phosphatase activity was stimulated by 5 mM Mn^{++} and inhibited by 2 mM ATP (Fig. 20B).

f. Ethanol Treatment of Cytosol Protein Phosphatases from Renal Medulla

Phosphatases Pa and Pb were pooled, treated with ethanol and rechromatographed on a DEAE-cellulose column. Pa was eluted at the same

Table 16: The Purification of Membrane-Bound Protein Phosphatase Ma.*

	Specific Activity (U/mg)	Recovery	Purification	Total Activity (U)
PPM	0.64	--	1	107
1% Triton X-100	4.12	92%	6.4	98
DEAE-cellulose DE-52	9.71	64.7%	15	69
Sephadex G-200	24.5	49%	38	52
DEAE-Sephadex A-50	42	34%	65	36

*The purified fractions were assayed by the standard protein phosphatase assay method described in the Experimental Procedure section. [^{32}P]-histone was used as substrate. One unit (U) of protein phosphatase activity is defined as the amount of enzyme which catalyzes the removal of 1 nmole of orthophosphate from [^{32}P]-histone per min under the standard assay condition.

KCl concentration (175 mM) as in the original chromatographic run; the phosphatase activity originally associated with Pb, however, no longer eluted at a KCl concentration of 240 mM but rather at one of 175 mM, where Pa was eluted (Fig. 21A). Combining Pa with ethanol-treated Pb and then rechromatographing on DEAE-cellulose resulted in a single peak eluted at the same salt concentration (175 mM) (Fig. 21B). Furthermore, gel filtration of pooled Pa and treated Pb through a Sephadex G-100 column also yielded a single peak of phosphatase activity, as did chromatography on a DEAE-cellulose column of ethanol-treated Pb and untreated Pa (Fig. 22). The single peak was characterized by a molecular weight of 34,000 daltons. Ethanol treatment of Pa did not result in any change in phosphatase activity, whereas ethanol treatment of Pb resulted in a 90% increase in activity (Table 17).

g. Comparison of Cytosol and Membrane-Bound Protein Phosphatases

When compared to the cytosol phosphatase Pa and Pb the membrane phosphatase fractions Ma and Mb have similar chromatographic properties and similar behavior on ethanol treatment. When the cytosol was kept at 4°C for one week, the phosphatase fraction Pb disappeared, i.e., it was no longer present in the elution profile from a DEAE-cellulose column. All the foregoing results indicate that the minor cytosol phosphatase peak, Pb, and the membrane-bound phosphatase peak, Mb, can shift to a small molecular weight form which is identical to the major cytosol phosphatase peak, Pa, and the membrane-bound phosphatase peak, Ma. These changes are accompanied by an increase in the enzymatic activity of Pb and Mb. Pb and Mb are unstable under conditions of prolonged storage at 4°C and form of Pb and Mb, respectively, as they break down.

Table 17: Ethanol Treatment of Renal Protein Phosphatase.*

	Pa	Pb	Ma	Mb
Untreated Phosphatase Activity (U/mg)	4.76	2.34	9.51	2.08
Ethanol Treatment Activity (U/mg)	5.10	5.62	10.4	4.67

*The assay was carried out by the standard phosphatase assay method described in the Experimental Procedure section. [^{32}P]-histone was used as the substrate, and ethanol-treated or untreated renal protein phosphatase were the enzymes.

h. Separation of Cytosol Protein Phosphatases from Renal Cortex

The cytosol fraction and the cytosol protein phosphatases from canine renal cortex was obtained by the same cell fractionation methods and the same molecular fractionation and chromatographic procedures used previously for renal medulla. A major peak and a minor peak were eluted at the same KCl concentrations, namely, 175 mM and 240 mM, respectively (Fig. 23A). The activity of the minor peak was generally less than 10% of the total cytosol phosphatase activity of renal cortex, and this peak was occasionally absent entirely in some preparations. Rechromatography of a combination of the major cytosol peak from cortex with the major cytosol peak Pa from medulla on a DEAE-cellulose column resulted in a single peak (Fig. 24) which also remained unchanged after gel filtration through a Sephadex G-100 column (Fig. 25). Thus, the major cytosol peaks of protein phosphatase activity (Pa) appeared to be identical in the renal cortex and renal medulla (Fig. 24A, Fig. 25). The same methodology was also used to show that the minor cytosol peaks of protein phosphatase activity (Pb) appeared to be identical in the cortex and medulla (Fig. 24B). In addition, a Mn^{++}-dependent phosphatase was eluted from the cytosol fraction of renal cortex on a DEAE-cellulose column (Fig. 23B). The enzymatic activity of this fraction was detected only when 5 mM $MnCl_2$ was present in the assay mixture. This enzyme, which accounted for approximately 10% of the total cytosol protein phosphatase activity, was eluted at a KCl concentration of 100 mM. No Mn^{++}-dependent enzyme was detected in the corresponding cytosol fraction from renal medulla.

i. Purification of Cytosol Protein Phosphatase Pa

The major cytosol protein phosphatase Pa was purified approxi-

mately 283-fold relative to crude cytosol (Table 18) by the following steps:

1) Homogenization of tissue
2) Ammonium sulfate fractionation
3) DEAE-Sephadex A-50 ion-exchange chromatography
4) Ethanol treatment
5) Sephadex G-100 gel filtration
6) DEAE-Sephadex A-50 ion-exchange chromatography

j. Characteristics of Cytosol and Membrane-Bound Protein Phosphatases from Canine Renal Medulla

(1) The pH Optimum for Protein Phosphatase

As shown in Fig. 26, the pH optimum for protein kinase from the crude cytosol fraction from renal medulla and cortex is 6.8-7.2. In this range of optimal pH, 5 mM Mn^{++} stimulated enzymatic activity about 80%. In the presence of 2 mM ATP, the pH optimum for phosphatase activity was shifted to 7.8 (7.5-8); at pH 7.0, ATP inhibited the enzymatic activity of the cytosol phosphatase Pa from renal medulla by 50%. Membrane-bound phosphatase, Ma, also exhibited the same pH optimum and ATP sensitivity.

(2) The Molecular Weight

The molecular weight of the major cytosol phosphatase Pa (Mr = 35,000) is in the same range of the major membrane-bound phosphatase Ma (Mr = 33,000) (Fig. 27), and that of cytosol phosphatase Pb (Mr = 100,000) is in the same range of the minor membrane-bound phosphatase Mb (Mr = 108,000) (Fig. 28). The result indicates the similar nature of cytosol and membrane phosphatases.

Table 18: Renal Cytosol Protein Phosphatase Pa Purification.*

		Total Protein (mg)	Specific Activity (U/mg)	Recovery	Purification
1.	Crude Extract	32,700	1.12	100%	1
2.	Ammonium Sulfate Fractionation (55%)	12,208	2.61	87%	2.3
3.	DEAE-Sephadex A-50 Fractionation	5,715	4.87	76%	4.3
4.	Ethanol Treatment	2,249	8.97	54%	8
5.	DEAE-Cellulose Chromatography	739	21.8	44%	19.5
6.	Sephadex G-100 Chromatography	196	110.6	24%	98.8
7.	DEAE-Sephadex A-50 Chromatography	17.4	317	14%	283

*The assays of purified fractions were carried out by the standard phosphatase assay method described in the Experimental Procedure section. [^{32}P]-histone was used as the substrate. One unit (U) of protein phosphatase activity is defined as the amount of enzyme which catalyzes the removal of 1 nmole of orthophosphate from [^{32}P]-histone per minute under the standard assay condition.

(3) Effect of Divalent Cations on Protein Phosphatase Activity

As indicated in Table 17, a 60-80% stimulation of the activities of both cytosol phosphatases Pa and Pb and both membrane phosphatases Ma and Mb occurs in the presence of Mg^{++}. Mn^{++} is also associated with a substantive (80-100%) stimulation of these enzymes. The concentration of either Mg^{++} or Mn^{++} required for maximum stimulation is approximately 5 mM. The Mn^{++}-dependent enzyme found only in the cortex was totally inactive in the absence of Mn^{++}.

On the other hand, Zn^{++}, Cu^{++}, and Ca^{++} inhibit all renal cortical and medullary cytosol and membrane-bound phosphatases (Table 19). Zn^{++} is the most potent inhibitor; at a concentration of 2 mM, Zn^{++} evoked a 95% inhibition of the crude membrane-bound phosphatase. The membrane phosphatases Ma and Mb proved to be more resistant to inhibition by Zn^{++} in their solubilized states than they were in their more native membrane-bound states. Table 17 shows the stronger inhibitory action of Zn^{++} compared to Cu^{++}.

(4) Effects of ATP, ADP, AMP, Cyclic AMP and Cyclic GMP

ATP, ADP, and AMP inhibited the activities of all renal phosphatases. At a ligand concentration of 2 mM, the enzymatic activity was inhibited 40-50% by ATP, 25-40% by ADP, and 10-20% by AMP; thus, the inhibitory potency of the adenine nucleotides follows the sequence: ATP, ADP, AMP (Table 20).

Inorganic phosphate (Pi) and particularly pyrophosphate (PP_i) also inhibited all renal phosphatases. In the presence of 2 mM PP_i, enzymatic activity was inhibited 70-80%, whereas in the presence of 20 mM Pi, it was inhibited only 40-50%.

Table 19: The Effect of Divalent Ions on Renal Protein Phosphatase Activity.*

		Specific Activity (U/mg)				
		Control	Mg^{++} (5 mM)	Mn^{++} (5 mM)	Cu^{++} (2 mM)	Zn^{++} (2 mM)
Unsolubilized Membrane-Bound Phosphatase		0.461	0.714	0.978	0.084	0.05
Membrane Phosphatase	Ma	14.21	20.2	24.3	3.69	5.2
	Mb	8.91	12.3	14.5	4.21	3.2
Cytosol Phosphatase	Pa	20.3	30.4	36.3	11.3	5.1
	Pb	14.7	17.6	24.1	6.7	4.3
Cortex Mn^{++}-Dependent Phosphatase		--	0.21	2.35	--	--

*The assay was carried out by the standard protein phosphatase assay method described in the Experimental Procedure section. [^{32}P]-histone was used as the substrate.

Table 20: The Effect of ATP, ADP, AMP, Pyrophosphate and Phosphate on Renal Protein Phosphatase Activity.*

		Specific Activity (U/mg)					
		Control	ATP (2 mM)	ADP (2 mM)	AMP (2 mM)	Pyrophosphate (2 mM)	Phosphate (2 mM)
Membrane-Bound Phosphatase (unsolubilized)		0.461	0.283	0.312	0.401	0.136	0.237
Membrane-Bound Phosphatase	Ma	14.21	8.12	9.31	12.01	3.32	7.1
	Mb	8.91	5.32	5.94	7.83	2.63	4.22
Cytosol Phosphatase	Pa	20.3	10.3	13.6	17.6	5.61	10.1
	Pb	14.7	8.1	9.61	12.3	4.80	7.6

*The assay was carried out by the standard protein phosphatase assay method described in the Experimental Procedure section.

The cyclic nucleotides, cyclic AMP and cyclic GMP, neither stimulated nor inhibited any of the renal medullary or cortical, membrane-bound (native or solubilized) or cytosol protein phosphatases (Table 18).

(5) K_m Values Related to Protein Phosphatase Activity

The K_m values with either [^{32}P]-histone or [^{32}P]-casein as substrates for various protein phosphatases are shown in Table 19. When histone is used as substrate, the K_m values for solubilized membrane phosphatases Ma and Mb are lower than the corresponding ones for cytosol phosphatases Pa and Pb; when casein is used as substrate, the corresponding K_m values are similar for the membrane-bound phosphatases Ma and Mb and the cytosol phosphatases Pa and Pb. Under all conditions studied, however, the K_m values for phosphatases Ma and Mb were lower when the enzymes were in their native membrane-bound states than when they were in solubilized states.

k. Molecular Weights of Renal Membrane-Bound Phosphatases Ma and Mb and Cytosol Phosphatases Pa and Pb

The molecular weights of renal membrane-bound phosphatases Ma and Mb were estimated from gel filtration on Sephadex G-100 and G-200 columns, as shown in Figs. 27 and 28, and those of the cytosol protein phosphatases Pa and Pb were calculated from the sedimentation coefficient ($S_{20,W}$) value derived from sucrose gradient centrifugation (Fig. 30) and the Stokes radius obtained from gel filtration (Fig. 29) according to the following equation:

$$M = 6\pi\eta NaS/(1 - v\rho)$$

where a = the Stokes radius, S = the sedimentation coefficient, N =

Avogadro's number, η = the viscosity of water, ρ = the density of water, and v = the partial specific volume of the protein (assumed to be 0.725 for protein phosphatases). $S_{20,W}$ values were determined by sucrose density gradient centrifugation as described by Martin and Ames (212). The molecular weights are summarized as follows:

	Stokes radius (Å)	$S_{20,W}$ (S)	Molecular weight (dalton)
Cytosol Phosphatas			
Pa	24	3.5	35,000
Pb	45	5.4	100,000
Membrane Phosphatase			
Ma	24		33,000
Mb	45.5		108,000

4. Phosphorylation and Dephosphorylation of Plasma Membrane

a. Phosphorylation of Papillary Plasma Membranes (PPM)

Incubating PPM with the assay medium results in endogenous membrane phosphorylation; in the presence of cyclic AMP, the phosphorylation increased 50% to 100% over basal levels (Table 7). When membranes were solubilized with Triton X-100, cyclic AMP-dependent phosphorylation of PPM increased 200% over basal levels (the basal activity also increasing by 50% to 80% after solubilization). On the other hand, when either highly purified type II protein kinase from beef heart or renal tissue was used, the phosphorylation increased 200% in the case of the unsolubilized membranes and 500% in the case of the solubilized membrane.

For unsolubilized membranes in the absence of cyclic AMP, 15

Table 21: Km Value and Molecular Weight of Renal Protein Phosphatase

	Km for [^{32}P]-Histone (M)	Km for [^{32}P]-Casein (M)	M.W. (dalton)
Cytosol Phosphatase			
Pa	5×10^{-4}	2.3×10^{-5}	35,000
Pb	1×10^{-4}	2.9×10^{-5}	100,000
Membrane-Bound Phosphatases			
Ma	5.2×10^{-5}	4.5×10^{-5}	33,000
Mb	2.0×10^{-5}	2.5×10^{-5}	108,000
PPM-Associated Phosphatase	8.3×10^{-6}	5.6×10^{-6}	---

minutes were required for the phosphorylation reaction to reach its maximum, whereas in the case of cyclic AMP-dependent phosphorylation, more than 20 minutes were required before the reaction reached its maximum. As shown in Fig. 31, when purified catalytic subunit of protein kinase was used to phosphorylate membranes, only 5 minutes were required for the reaction to reach its maximum for either solubilized or unsolubilized plasma membranes. When purified plasma membrane-bound protein kinase (MPK II) was used, the reaction was slower and 15 min were required before the reaction reached its maximum. When histone was used as the substrate for MPK II, the phosphorylation attained its maximum in 5 minutes (Fig. 32). In contrast, the phosphorylation of PPM catalyzed by the catalytic subunit of cytosol protein kinase CPK II was quite rapid (Fig. 32). When membrane-bound protein kinase was used to catalyze the phosphorylation of histone, the reaction attained its maximum after 20 min (Fig. 32). The reaction involving cytosol protein kinase and membrane-bound substrate also proved to be relatively slow; on the other hand, the purified catalytic subunit of protein kinase can react with membrane-bound substrate rapidly. The solubilization of membrane resulted in increasing the accessibility of the substrate to the active site of the enzyme, which not only accelerated the reaction but also increased the level of substrate phosphorylation.

b. Dephosphorylation of Membrane and Membrane Phosphatase

Previous results indicated that plasma membrane has both membrane-bound protein kinases and phosphatases. *In vivo*, the state of phosphorylation of the plasma membrane will depend on the balance between

the rates of phosphorylation and dephosphorylation whether or not cyclic AMP is present. *In vitro*, in order to achieve maximum phosphorylation, it is necessary to inhibit the activity of phosphatases by addition of NaF, Zn^{++}, pyrophosphate or heat-stable phosphatase inhibitor. Sodium fluoride can inhibit phosphatase activity without affecting protein kinase activity. Zn^{++}, however, not only inhibited phosphatase activity but also inhibited phosphorylation of membrane. As shown in Table 22, in the presence of 20 mM NaF, the phosphorylation over a period of 5 min increased 30%, whereas Zn^{++} reduced the level of phosphorylation by 20%. Pyrophosphate proved to be a strong inhibitor of renal phosphatases but did not affect the activity of renal protein kinases.

The control of the state of phosphorylation of PPM depends on three membrane-bound enzymes, namely, cyclic AMP-dependent protein kinase, protein phosphatase and phosphodiesterase. *In vivo*, without addition of theophylline, the cyclic AMP-dependent phosphorylation reached a maximum level in 5 min and decreased more rapidly than when theophylline was added to the system. This effect diminished when protein kinase catalytic subunit was used to catalyze the phosphorylation reaction; in this case, maximum phosphorylation was attained in 10 min (Fig. 33). When the phosphatase activity was also inhibited, it reached a maintained stable state after 10 min (Fig. 34). In the presence of 20 mM NaF, cyclic AMP-dependent phosphorylation was increased by 40% with respect to the control in which NaF was absent and attained a maximal level in 5 minutes. On the other hand, the presence of 2 mM Zn^{++} inhibited 90% of the membrane-bound phosphatase

Table 22: The Effect of NaF, Zn^{++} and Theophylline on the Phosphorylation of Solubilized Papillary Plasma Membrane.*

	Specific Activity (mU/mg)			
	5 min		10 min	
	-cAMP	+cAMP 1 μM	-cAMP	+cAMP 1 μM
Control	4.8	8.5	5.1	10.1
+ 2 mM Zn^{++}	5.1	6.8	5.3	8.3
+ 20 mM NaF	5.8	11.2	6.0	12.9
+ 2 mM Theophylline	5.3	9.8	5.4	10.3

*The assay was carried out by the standard kinase assay method described in the Experimental Procedure section, except that the solubilized plasma membrane is used as substrate. The final concentration of membrane was 1.2 mg/ml. The incubation time was 5 min or 10 min at 30°C. One unit (U) of activity is defined as the amount of enzyme necessary to catalyze the transfer of 1 nmole of orthophosphate from [γ-^{32}P]ATP to the membrane preparation per minute.

activity, resulting in a 50% reduction in the level of membrane phosphorylation (Fig. 34).

Fig. 35 shows that isolated membrane-bound phosphatase Ma is capable of dephosphorylating phosphorylated PPM. This dephosphorylation of PPM is relatively slow compared to that of phospho-casein and phospho-histone under similar conditions. The cytosol phosphatase also catalyzed the dephosphorylation of PPM relatively slowly (Fig. 35).

c. SDS Polyacrylamide Gel Electrophoresis of the Phosphorylated Papillary Plasma Membrane

The phosphorylation of PPM mainly occurred in the regions of Mr = 100,000, 50,000, and 38,000. The major peak is at M_r = 50,000 region (P-50). The phosphorylation of this peak is greater than that of the other peaks. The presence of cyclic AMP (10^{-5} M) slightly increased the phosphorylation of the minor peaks at Mr = 100,000 and Mr = 38,000. Cyclic AMP at this concentration also increased the phosphorylation of the major peak protein (M_r = 50,000) by 20% to 30% as shown in Figure 36. However, this cyclic AMP-induced increase in phosphorylation was observed only when the phosphatase inhibitors (NaF, 20 mM or $ZnCl_2$ 2mM) were added to the system.

d. Cyclic AMP-Dependent Dephosphorylation of Papillary Plasma Membrane

Fig. 37A shows that the phosphorylation of the major peak (M_r = 50,000) in the presence of 1 μM cyclic AMP but in the absence of phosphatase inhibitors was substantially decreased in comparison to the phosphorylation of PPM in the absence of cyclic AMP. On the

other hand, when phosphatase inhibitor was added to the reaction, the major peak (M_r = 50,000) was not dephosphorylated either in the presence or absence of cyclic AMP.

To summarize the foregoing results:

1. In the absence of cyclic AMP

   a) Protein P-50 (M_r = 50,000) was phosphorylated.

   b) The phosphorylation of the protein P-49 was not affected by phosphatase (i.e., the presence of the phosphatase inhibitors, NaF, did not result in an increase in the phosphorylation of this protein).

2. In the presence of cyclic AMP (10 μM)

   a) The phosphorylation of P-50 was enhanced provided that there was no phosphatase activity in the system.

   b) P-50 was dephosphorylated if phosphatase activity was present in the system (cyclic AMP-dependent dephosphorylation).

e. Autophosphorylation and Cyclic AMP-Dependent Dephosphorylation of Membrane Protein Kinase MPK II

(1) Autophosphorylation of MPK II

Polyacrylamide gel electrophoresis of purified membrane-bound protein kinase (MPK) II shows that the following three peaks were located in the same region (Fig. 38):

a) Cyclic AMP-dependent protein kinase activity (using histone as substrate;

b) The autophosphorylated holoenzyme as indicated by the ^{32}P label;

c) [^{3}H]-cyclic AMP binding protein.

When the cyclic AMP (10 μM)-treated MPK II was subjected to gel electrophoresis, the [^{3}H]-cyclic AMP binding peak and the ^{32}P-labeled autophosphorylation peak (i.e., the regulatory subunit of protein kinase) were located at the same region.

In the presence of 10 μM cyclic AMP the holoenzyme, MPK II, dissociated into its regulatory and catalytic subunits. This dissociation resulted in a shifting of the peak of autophosphorylation from that of the holoenzyme to that of a smaller molecule which superimposes on the ^{3}H peak of the [^{3}H]-cyclic AMP bound to the regulatory subunit (Fig. 39). Therefore, because the molecular weight of regulatory subunit of MPK II is approximately 50,000 (Fig. 40A, B), these findings indicate, among other things, that the autophosphorylation of MPK II is located in the regulatory subunit of the enzyme.

(2) Cyclic AMP-Dependent Dephosphorylation of MPK II

Figure 41 shows that the autophosphorylation of MPK II, CPK II and renal collecting duct epithelial cell plasma membrane are all located at the same region of molecular weight (Mr = 50,000) (P-50). The dephosphorylation of this phosphoprotein occurs only in the presence of cyclic AMP. In other words, the dephosphorylation of protein P-50 occurs only when the holoenzyme is dissociated into its regulatory and catalytic subunits. Without this dissociation, even in the presence of protein phosphatase, the dephosphorylation of P-50 does not occur. Since the protein phosphatase added to the assay is not a cyclic AMP-dependent enzyme (i.e., the protein phosphatases, Ma and Pa, showed no dependence on cyclic AMP for the dephosphorylation of phospho-histone, phospho-casein and phosphorylase

b), it can be concluded that the regulation of this cyclic AMP-dependent dephosphorylation takes place at substrate level.

f. The Nature of Cyclic AMP-Dependent Dephosphorylation of Papillary Plasma Membrane (Autoradiography)

Figure 42 is a radioautograph of renal papillary plasma membrane phosphoproteins labeled with ^{32}P and separated on an SDS-polyacrylamide gel. These proteins were first phosphorylated in the presence of 5 mM Zn^{++}, a phosphatase inhibitor, and then exposed to cyclic AMP after removal of this inhibitor by chelation with EDTA. The feature of particular interest in this figure is the striking dephosphorylation of the protein band in the Mr = 50,000 region of the gel as compared to the same band in the control gel which displays the membrane phosphoproteins treated in an identical manner except for the omission of cyclic AMP. Molecular weight standards for this gel were BSA (Mr = 67,000) ovalbumin (Mr = 43,000) and cytochrome c (Mr = 17,000). A second band appears slightly below the major band in the (Mr = 50,000) region; the nature of this protein is unknown.

After solubilization of membrane protein and filtration through Sephacryl S-200, the eluates separated into three fractions: I (Mr = 20,000-70,000); II (Mr = 70,000-140,000); and III (Mr = 140,000-210,000). Each of these fractions was subjected to electrophoresis; the results showed that only fraction II which contained MPK II demonstrated autophosphorylation.

Fig. 1. Electrical Microscopic Study of Plasma Membrane (x 35,100)

Fig. 1. Electrical Microscopic Study of Papillary Plasma Membrane. The pellet of the plasma membrane fraction was fixed in 2% glutaraldehyde in 0.1 cacodylate buffer at pH 7.2 for 1 hour on ice and rinsed in cold buffer overnight. The pellet was then post-fixed in 1% osmium tetroxide in 100 mM phosphate buffer at pH 7.3 for 1 hour on ice. Dehydration in a graded series of ethanol (70%, 95% and 100%) solutions on ice was followed by propylene oxide and embedded in Epon 812.

Ultra-thin (silver) sections were cut on a Porter-Blum MT-2 Ultramicrotome with a diamond knife. Sections were stained with uranyl acetate (197) followed by lead citrate (198) and examined in a Philips 201 electron microscope. The photo was magnified 35,000 times.

Fig. 2

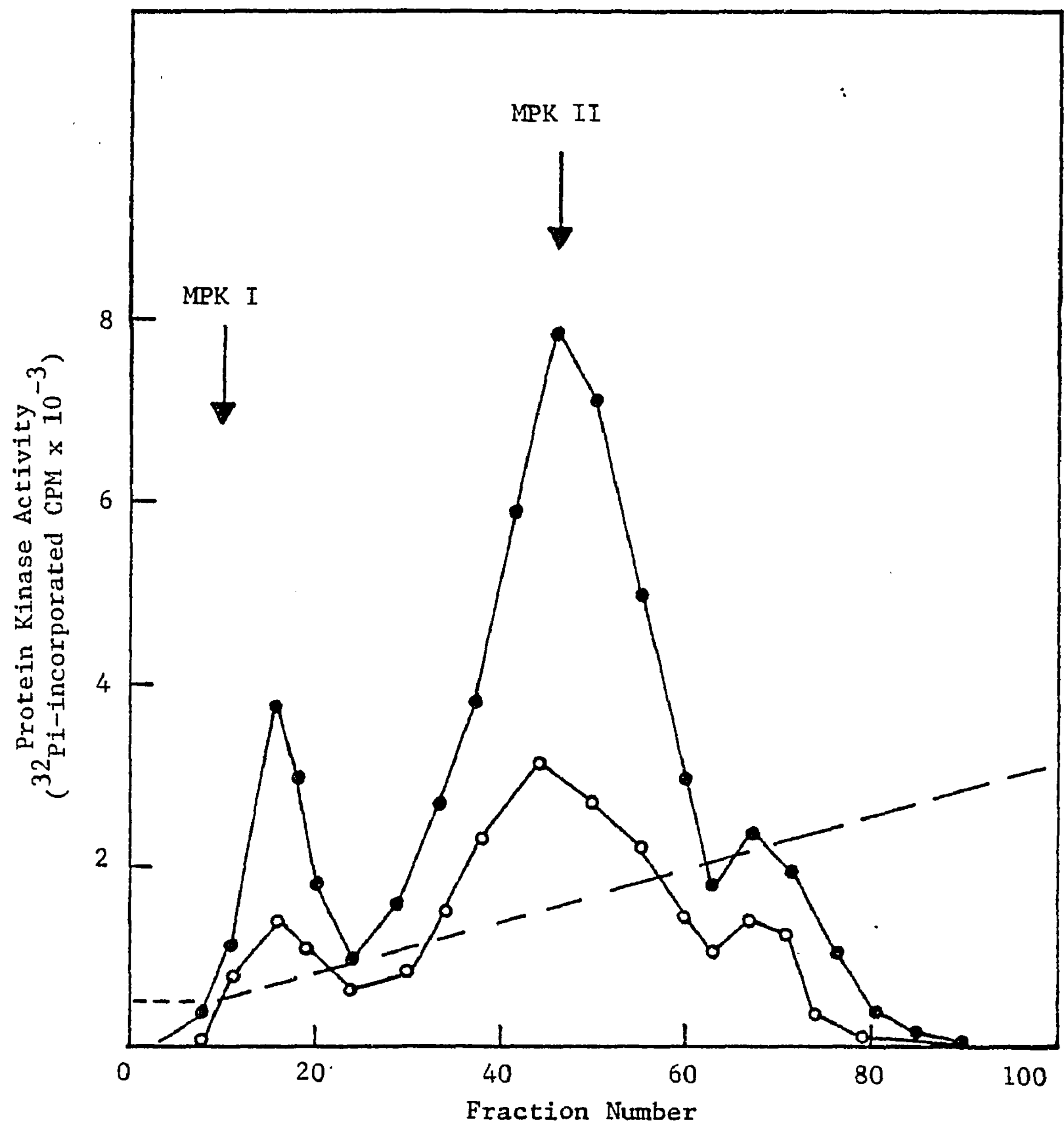


Fig. 2. Cyclic AMP-dependent protein kinase activity of the solubilized papillary plasma membrane fraction separated on DEAE-Sepharose CL-6B column chromatography. The solubilized membrane fraction (15 ml, 15 mg/ml) was applied on a DEAE-Sepharose CL-6B column (1.6 x 50 cm). After rinsing the column with 400 ml Buffer A-T, which was comprised of 20 mM Tris•HCl (pH 7.0), 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 0.5% Triton X-100. The enzyme was eluted with a linear gradient (20-400 mM) of KCl with a volume of 600 ml. One hundred and twenty fractions were collected. The cyclic AMP-dependent protein kinase activity was assayed in either the presence • or absence ∘ of 1 μM cyclic AMP. The conductivity of KCl gradient was measured by a conductivity meter and indicated by --- . The activity of protein kinase is indicated in CPM (counts per minute). The protein kinase activity was assayed on 10 μl aliquots of the indicated eluted fractions and according to the standard method described in the Experimental Procedure section. Histone was used as the substrate, and incubation was performed for 10 minutes at 30°C.

Fig. 3

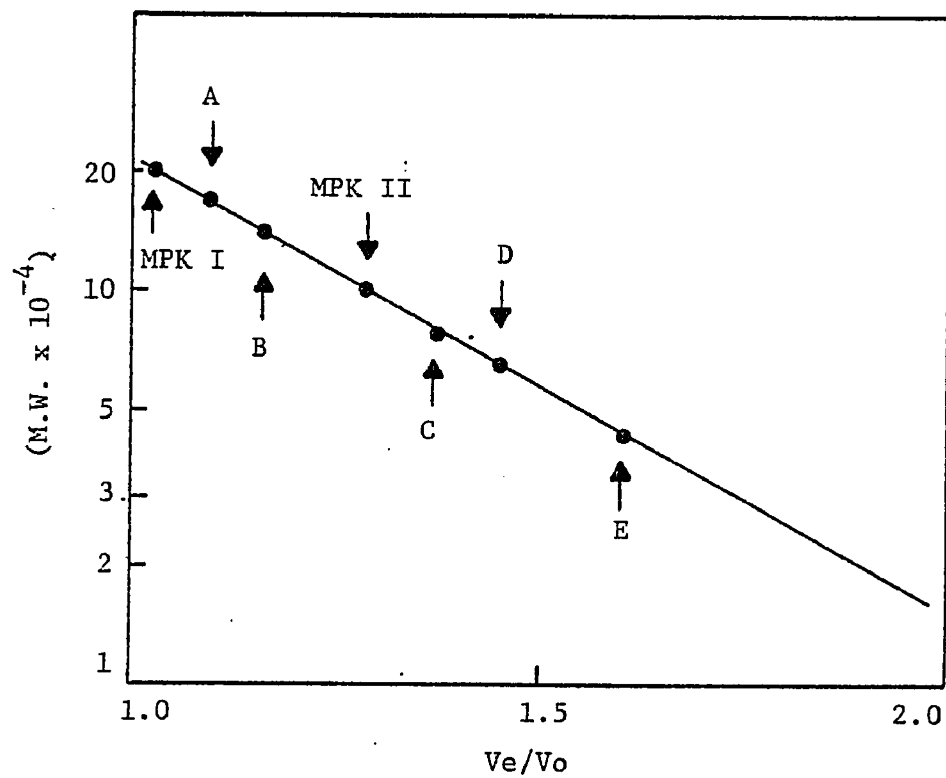


Fig. 3. Determination of the molecular weight of the membrane-bound protein kinases MPK I and MPK II by Sephadex G-200 gel filtration. The molecular weight of protein kinase MPK I and MPK II was determined by gel filtration on a Sephadex G-200 column (1.6 x 88 cm). The following proteins were used as standards for calibration:

A γ-globulin (Mr = 167,000)

B lactate dehydrogenase (Mr = 140,000)

C alcohol dehydrogenase (horse liver) (Mr = 80,000)

D bovine serum albumin (Mr = 67,000)

E ovalbumin (Mr = 43,000)

Fig. 4

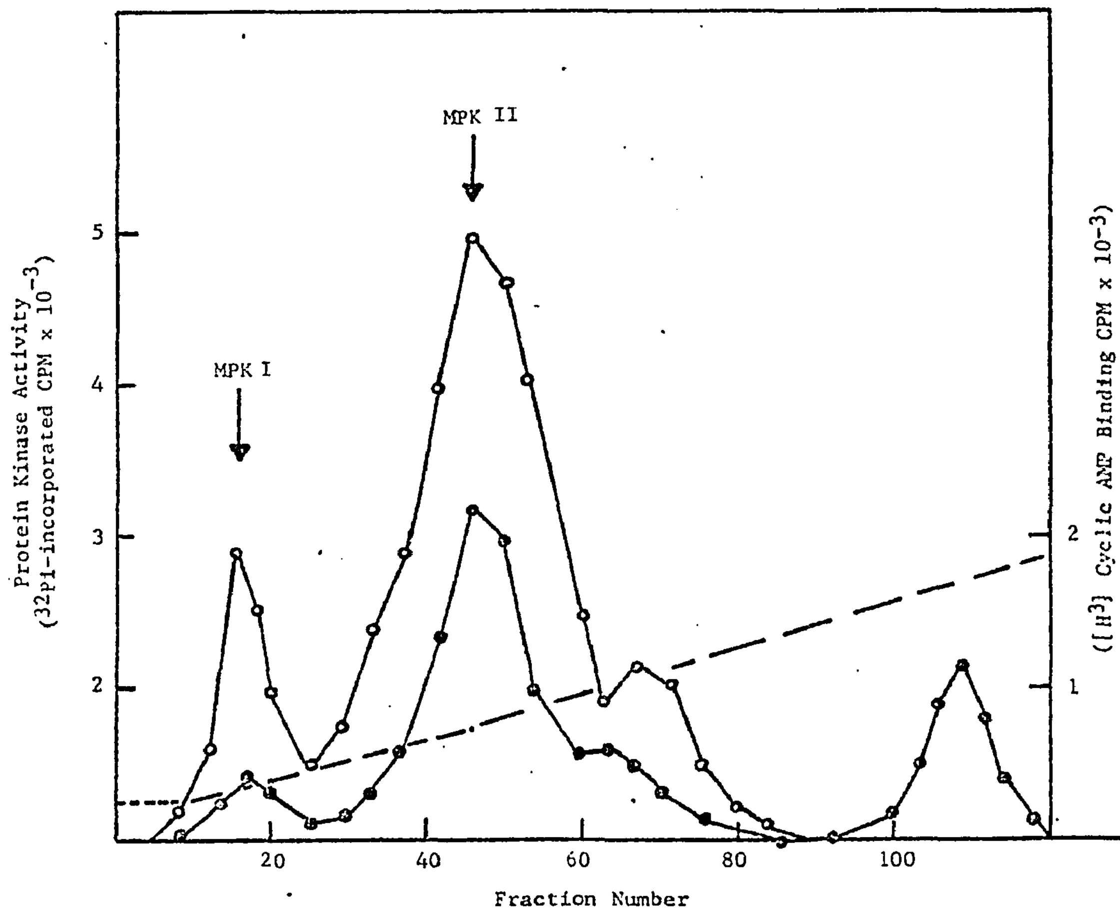


Fig. 4. Binding of [^{3}H]-cyclic AMP by renal medullary plasma membrane fraction separated on DEAE-Sepharose CL-6B column chromatography. The cyclic AMP binding assay was carried out by a modification of the Millipore filter technique of Gilman (200) described in the Experimental Procedure section. The assay mixture contained 20 mM Tris·HCl (pH 7.0), 4 mM magnesium acetate, 2 μg/ml of bovine serum albumin, 40 nM of tritium-labelled cyclic AMP (5 x 10^4 dpm/pmol), and the eluted aliquot in a final volume of 100 μl. The mixture was incubated at 23°C for 15 minutes. The cyclic AMP-dependent protein kinase activity is indicated by ∘, the [^{3}H]-cyclic AMP binding, by •, and the conductivity, by ---. The activities of protein kinase and AMP binding are indicated in CPM (counts per minute) in different scales on the graph.

Fig. 5

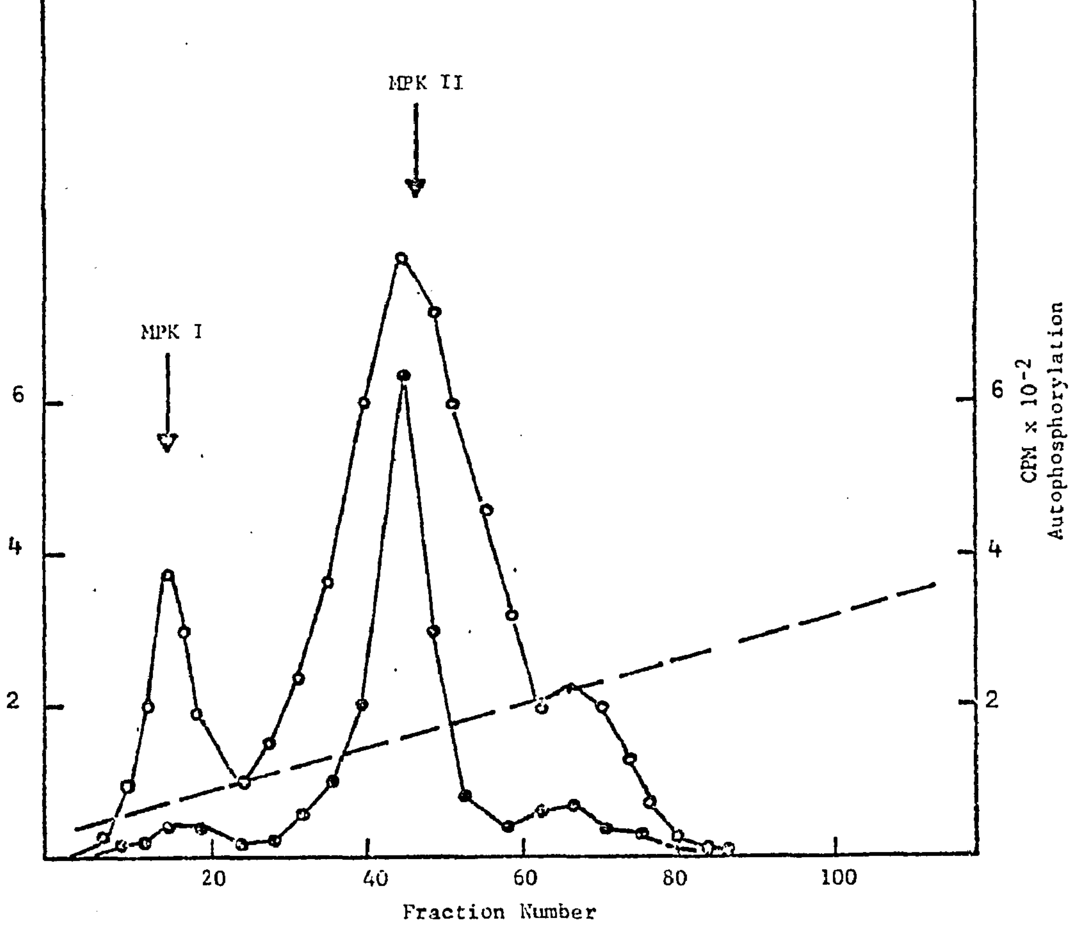


Fig. 5. Autophosphorylation of membrane-bound protein kinase separated on DEAE-Sepharose CL-6B Column Chromatography. The autophosphorylation was carried out according to Erlichman *et al.* (136), 20 μl of eluted aliquots were added to 50 μl of a solution containing 50 mM phosphate buffer (pH 7.1) 10 mM $MgSO_4$ and 50 μg of bovine serum albumin. The reaction was initiated by addition of 0.5 mM [γ-^{32}P]-ATP (200 cpm/pmol) and continued incubating for 10 min at 37°C. The reaction was stopped by addition of 250 μl of 0.1 M EDTA (pH 5.0). The contents of the tubes were then poured onto a millipore filter (HA 0.45 micron pore size) which quantitatively retains the enzyme and cyclic AMP binding protein. The filter was washed twice with 10 ml of the 0.1 M EDTA solution, then dried and assayed for ^{32}P in scintillation fluid containing 4 g of omniflor/liter of toluene. The cyclic AMP dependent protein kinase activity was assayed in the reaction mixture of 50 μl containing 1 μM cyclic AMP, 0.5 mM [γ-^{32}P]-ATP (100 cpm/pmol), 50 μg histone and one half dilution of 10 μl eluted aliquots of indicated fractions and according to standard method described in the Experimental Procedure section. Autophosphorylation was indicated by • and cyclic AMP dependent protein kinase activity by ∘ .

Fig. 6

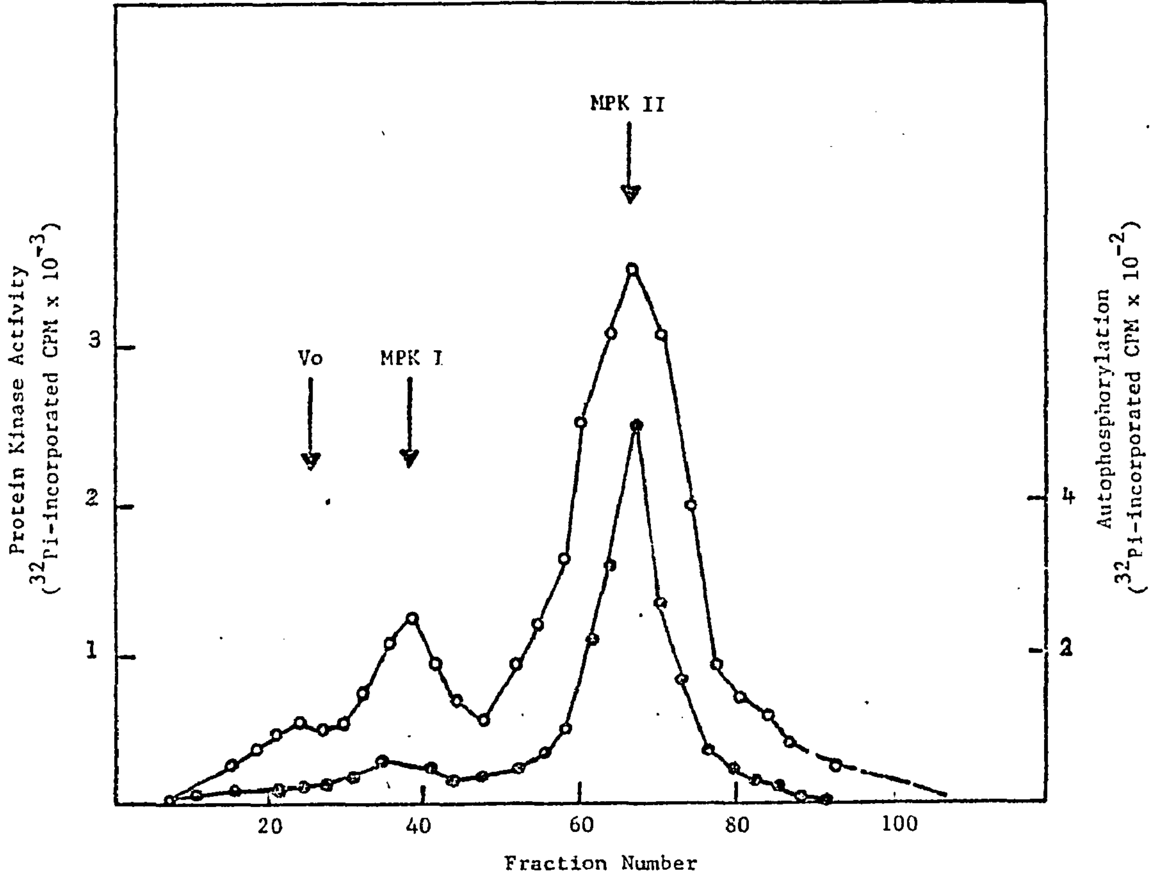


Fig. 6. Autophosphorylation of membrane-bound protein kinase separated by gel filtration of Sephacryl S-200. Solubilized plasma membrane (mg/ml, 8 mg/ml) was filtered through a Sephacryl S-200 (Pharmacia) column (1.6 x 75 cm) that was pre-equilibrated with Buffer A-T, which was comprised of 20 mM Tris•HCl (pH 7.0), 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 20 mM KCl and 0.5% Triton X-100. The column was eluted with the same buffer and each fraction contained 3.7 ml of eluate. The assay of autophosphorylation was carried out by the method of Erlichman et al. (136) on 10 µl aliquots of the indicated eluted fractions as described in the Experimental Procedure section. The cyclic AMP-dependent protein kinase activity was assayed by the standard assay method on 10 µl aliquots of one half of dilution of the indicated eluted fraction, as described. The autophosphorylation is indicated by •, and the cyclic AMP-dependent protein kinase activity, by ∘.

Fig. 7

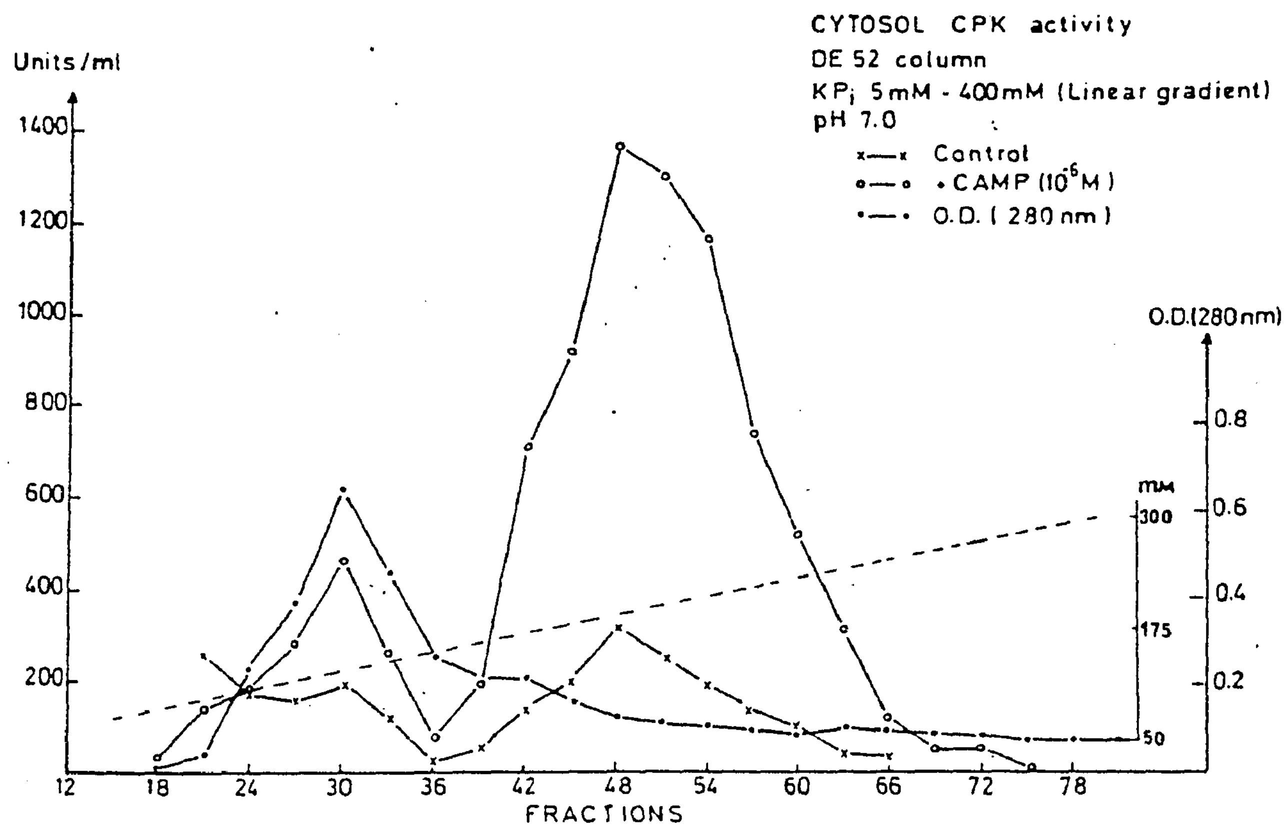


Fig. 7. Cyclic AMP-dependent protein kinase activity of renal medullary cytosol separated on DEAE-cellulose column chromatography. The cytosol fraction precipitated by 55% $(NH_4)_2SO_4$ (46 ml, 7.4 mg/ml) was applied to a DEAE-cellulose (Whatman DE-52) column (1.6 x 30 cm). Four hundred ml of 5 mM phosphate buffer (pH 7.0) was used to wash out the unabsorbed material. The enzyme was eluted with 600 ml of a linear gradient (5-400 mM) of potassium phosphate (pH 7.0). Ninety-five fractions were collected and each fraction containing 6.2 ml of eluate. The protein kinase assay was carried out on 10 μl aliquots of the indicated fractions and in accordance with the standard method described above. The protein kinase activity is indicated by ∘ in the presence of 1 μM cyclic AMP, and by X in the absence of cyclic AMP. The optical density at 280 nm is indicated by •, and the conductivity, by ---.

Fig. 8

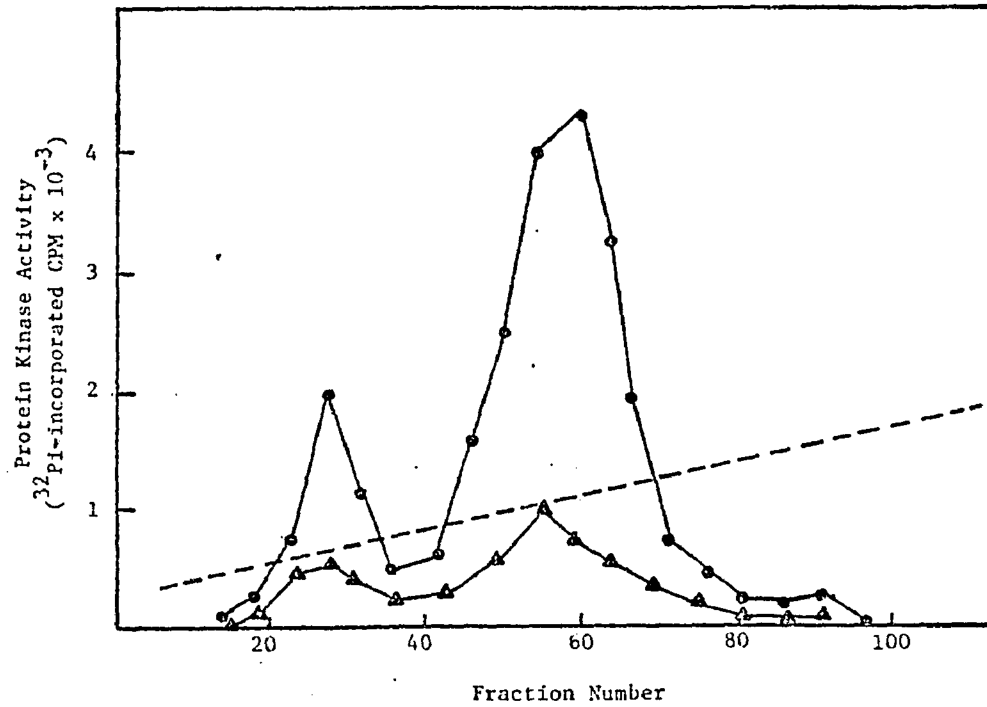


Fig. 8. Protein kinase activity of the cytosol fraction of renal cortex, separated on DEAE-cellulose column chromatography. The renal cortex cytosol fraction precipitated by 55% $(NH_4)_2SO_4$ (32 ml, 6.8 mg/ml) was applied to a column of DEAE-cellulose (Whatman DE-52) (2.6 x 45 cm) pre-equilibrated with 5 mM phosphate buffer (pH 7.0). The enzyme was eluted with 400 ml of a linear gradient (5-400 mM) of potassium phosphate (pH 7.0). The kinase activity on one fourth of dilution of 10 μl aliquots of the indicated eluted fractions was assayed with the standard method described above in the presence or absence of 1 μM cyclic AMP. The protein kinase activity is indicated by • in the presence of 1 μM cyclic AMP and by ▲ in the absence of cyclic AMP.

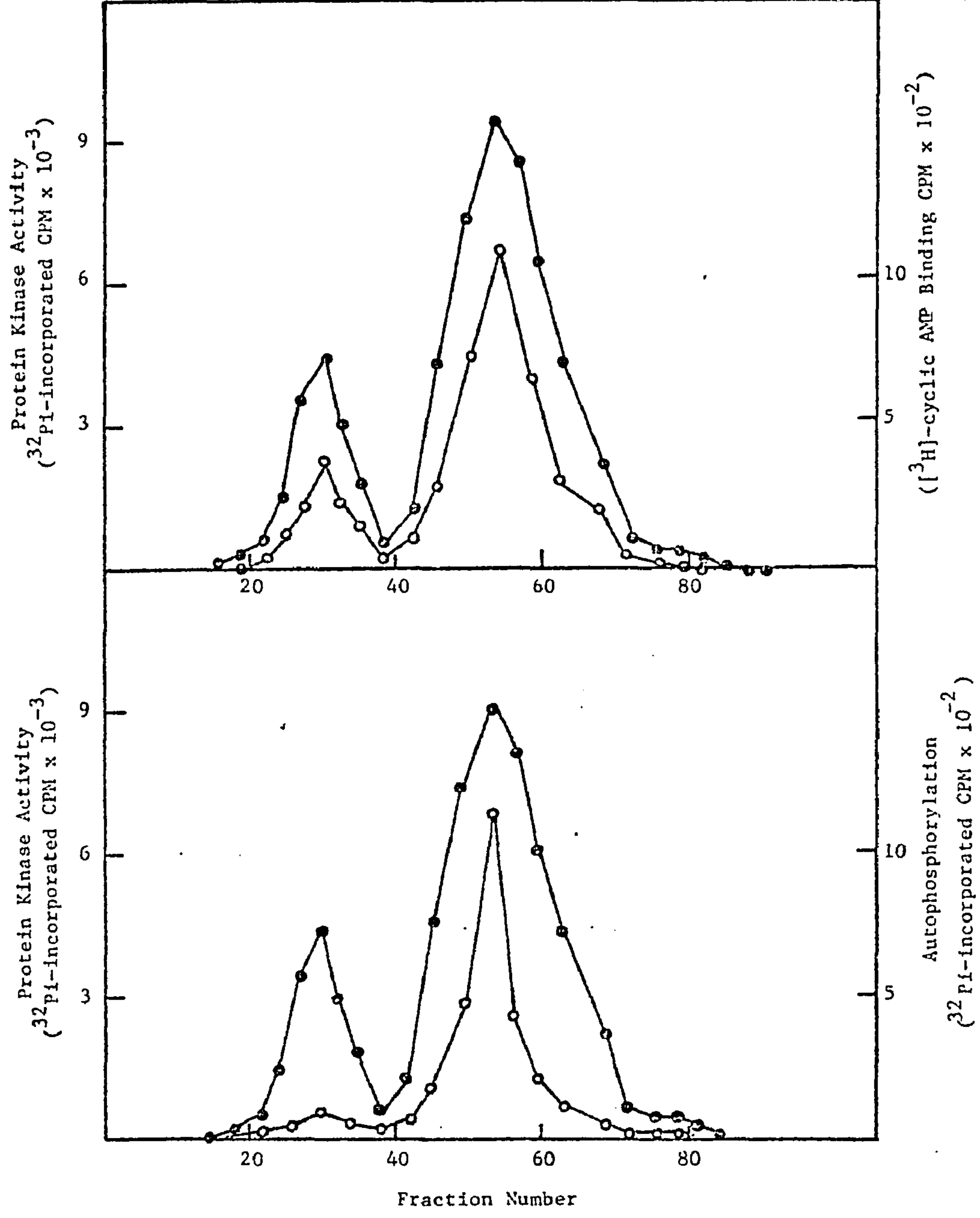


Fig. 9
Protein Kinase Activity
(^{32}Pi-incorporated CPM x 10^{-3})
3
6
9
([^{3}H]-cyclic AMP Binding CPM x 10^{-2})
5
10
20
40
60
80
Protein Kinase Activity
(^{32}Pi-incorporated CPM x 10^{-3})
3
6
9
Autophosphorylation
(^{32}Pi-incorporated CPM x 10^{-2})
5
10
20
40
60
80
Fraction Number

Fig. 9. [^{3}H]-cyclic AMP binding, autophosphorylation and protein kinase activity of cytosol fraction of whole kidney separated on DEAE-cellulose column chromatography. The renal cytosol fraction precipitated by 55% $(NH_4)_2SO$ (53 ml, 7.2 mg/ml) was applied to DEAE-cellulose (Whatman DE-52) (26 x 44 cm) pre-equilibrated with 5 mM phosphate buffer (pH 7.0). The enzyme was eluted with 600 ml of a linear gradient (5-400 mM) of potassium phosphate (pH 7.0). Fractions of 6.4 ml were collected. The [^{3}H] cyclic AMP binding and autophosphorylation activity were assayed on 10 µl aliquots of the indicated eluted fractions and protein kinase was assayed on one third of dilution of 10 µl aliquots by standard methods described in the Experimental Procedure section. The protein kinase activity is indicated by • for both parts A and B. The [^{3}H] cyclic AMP binding and the autophosphorylation is indicated by ∘ in parts A and B, respectively.

Fig. 10

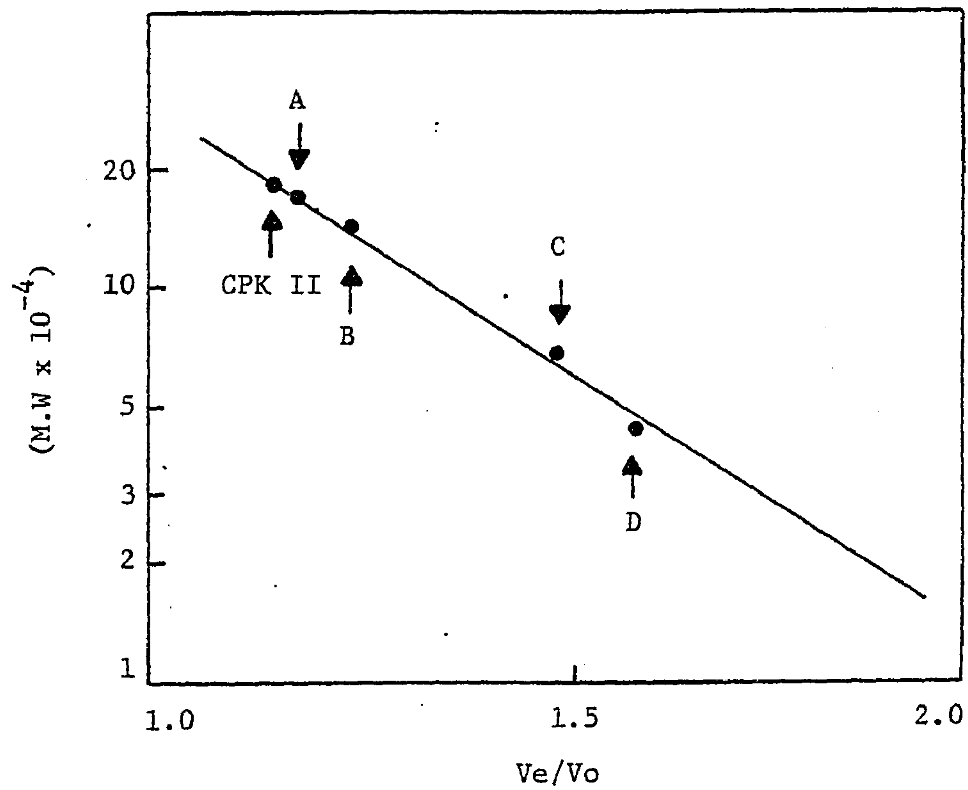


Fig. 10. Determination of the molecular weight of cytosol protein kinase CPK II by Sephadex G-200 gel filtration. The molecular weight of protein kinase CPK II was determined by gel filtration on a Sephadex G-200 column (2.5 x 60 cm). The following proteins were used as internal standards for calibration:

A γ-globulin (Mr = 167,000)

B lactate dehydrogenase (Mr = 140,000)

C bovine serum albumin (Mr = 67,000)

D ovalbumin (Mr = 43,000)

Fig. 11

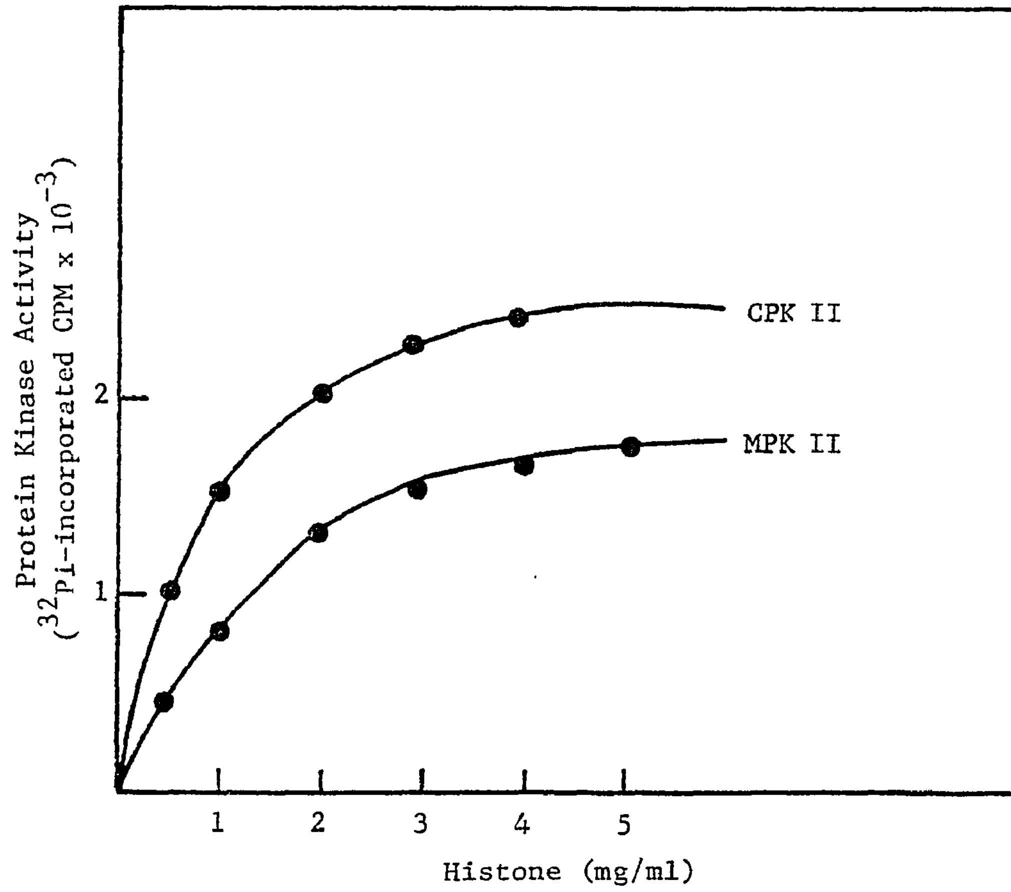


Fig. 11. The effect of concentration of substrate on protein kinase activity. The assay was carried out with the standard method described above. The reaction mixture of 100 μl contained 50 mM KPi (pH 7.0) 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]-ATP (50 cpm/pmol), 1 μM cyclic AMP, 25 μg of purified cytosol protein kinase CPK II and membrane-bound protein kinase MPK II, with histone concentrations ranging from 0.5 mg/ml to 5 mg/ml. The assay mixture was incubated for 10 min at 30°C. The upper line represents the substrate saturation curve for cytosol protein kinase CPK II and lower line that for membrane protein kinase MPK II.

Fig. 12

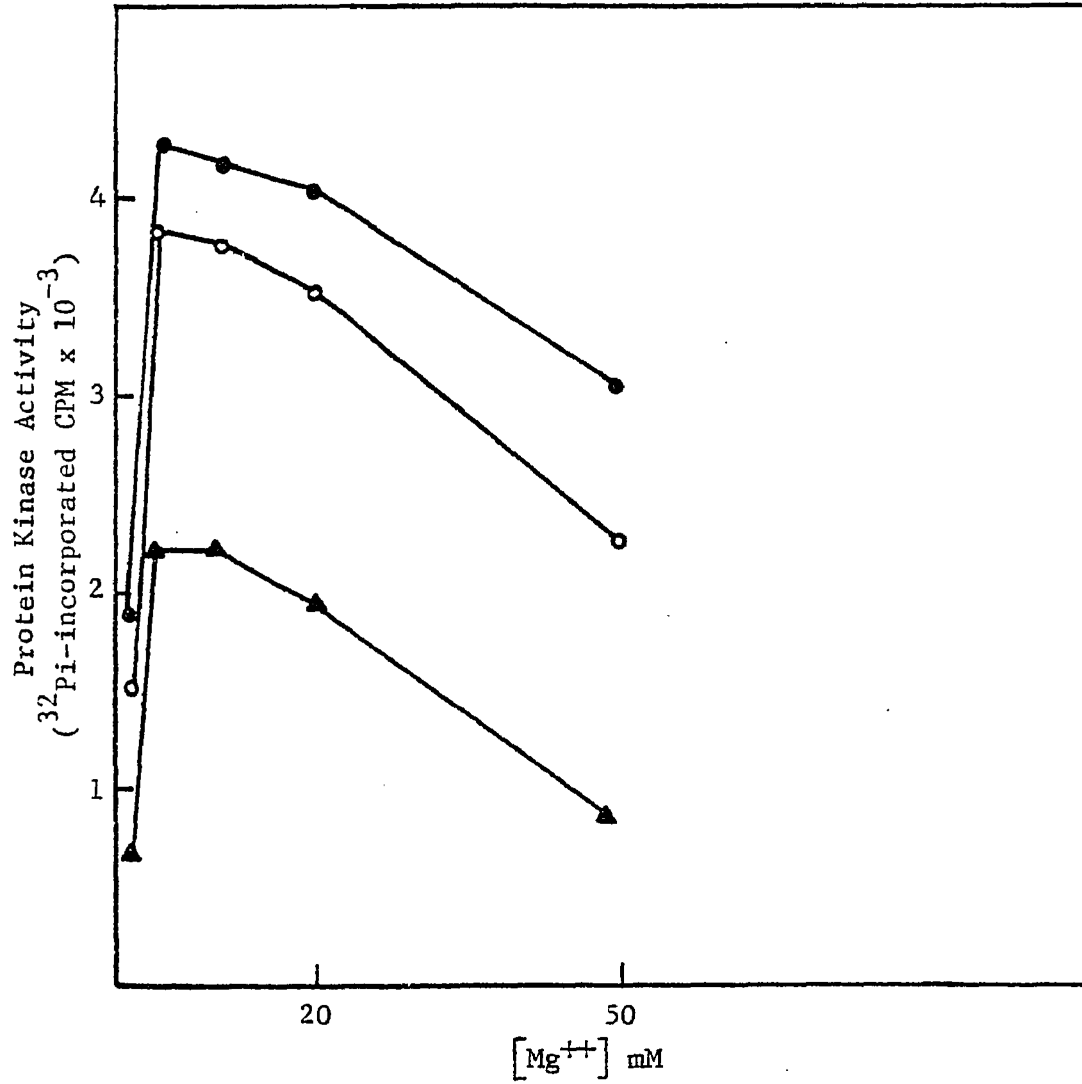


Fig. 12. The effect of Mg^{++} on protein kinase activity. The assay was carried out with the standard method described above. The reaction mixture of 50 µl contained 50 mM KPi (pH 7.0), 0.5 mM [γ-^{32}P]-ATP (50 cpm/pmol), 50 µg histone, 1 µM cyclic AMP, and 25 µg of reacting enzyme. The Mg^{++} concentration was varied from 2 to 50 mM. The incubation was 10 min at 30°C. The cytosol protein kinase (CPK II) activity is indicated by •, membrane protein kinase activity (MPK II), by ∘ and unsolubilized membrane kinase activity (MPK II), by ▲.

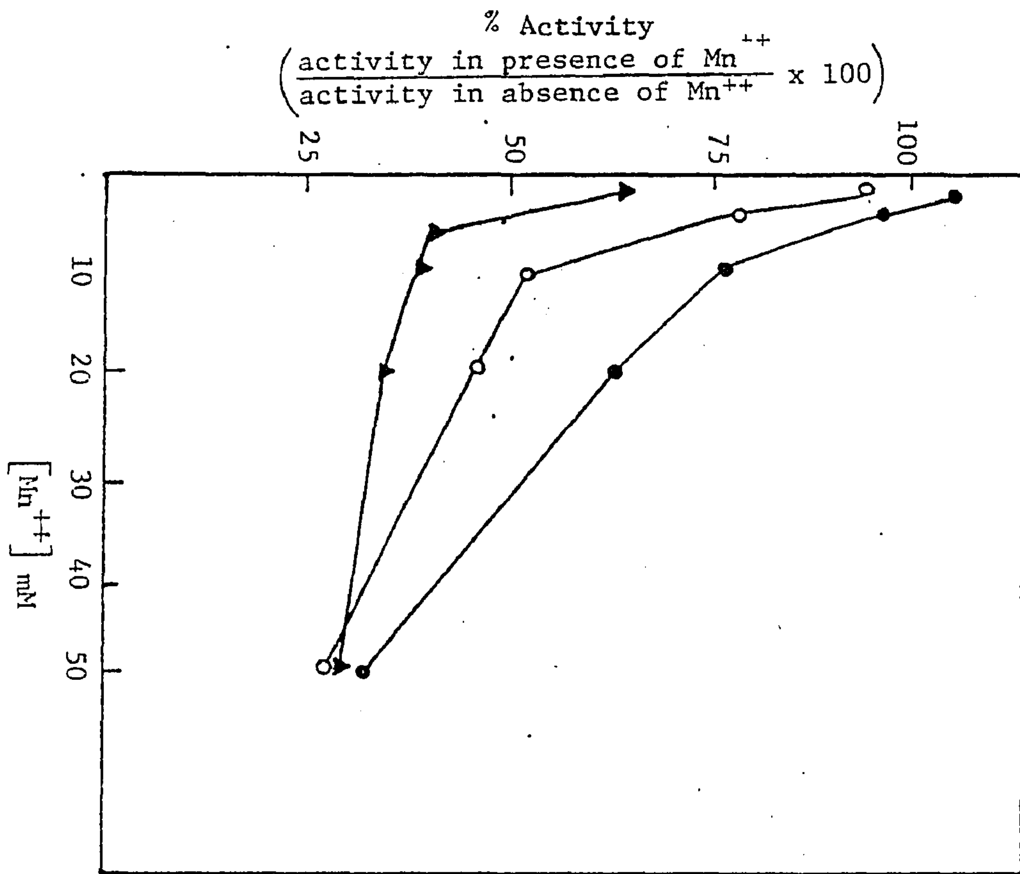


Fig. 13

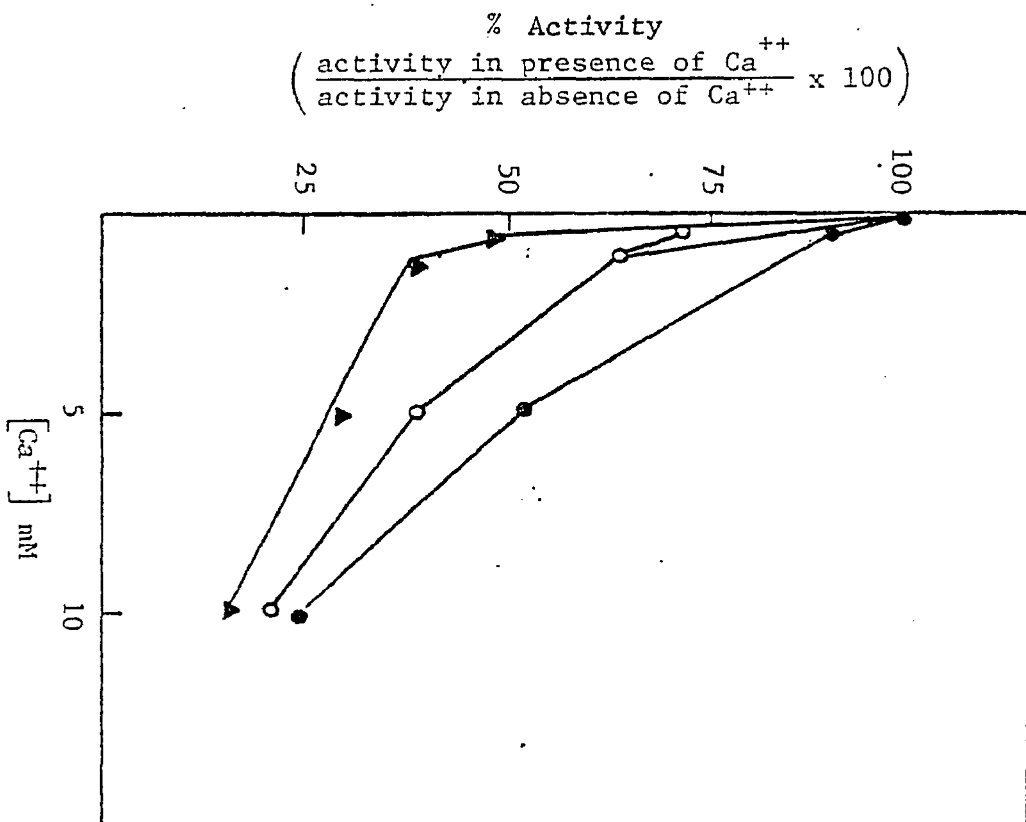


Fig. 14

Fig. 13. The effect of Mn^{++} on protein kinase activity. The assay was carried out by the standard assay method described above in the presence of 1 μM cyclic AMP with either no added $MnCl_2$ or the addition of $MnCl_2$ to a final concentration of 2 to 50 mM. Histone was used as the substrate at a concentration of 0.5 mM (50 cpm/pmol). The figure shows protein kinase activity in the presence of Mn^{++} expressed as a percentage of the protein kinase activity in the absence of Mn^{++} (control). The activity of cytosol protein kinase CPK II is indicated by •, membrane by protein kinase, MPK II by ∘, and unsolubilized membrane kinase by ▲.

Fig. 14. <u>The effect of Ca^{++} on protein kinase activity</u>. The assay was carried out by the standard method described above in the presence of 1 μM cyclic AMP with the addition of $CaCl_2$ to the final concentration from 0.5 to 10 mM. Histone was used as the substrate at a concentration of 1 mg/ml. [γ-^{32}P]-ATP was present at a concentration of 0.5 mM (50 cpm/pmol). The figure shows protein kinase activity in the presence of Ca^{++} expressed as a percentage of the protein kinase activity in the absence of Mn^{++} (control). The activity of cytosol protein kinase CPK II is indicated by •, membrane protein kinase MPK II by ∘ and unsolubilized membrane kinase by ▲.

Fig. 15

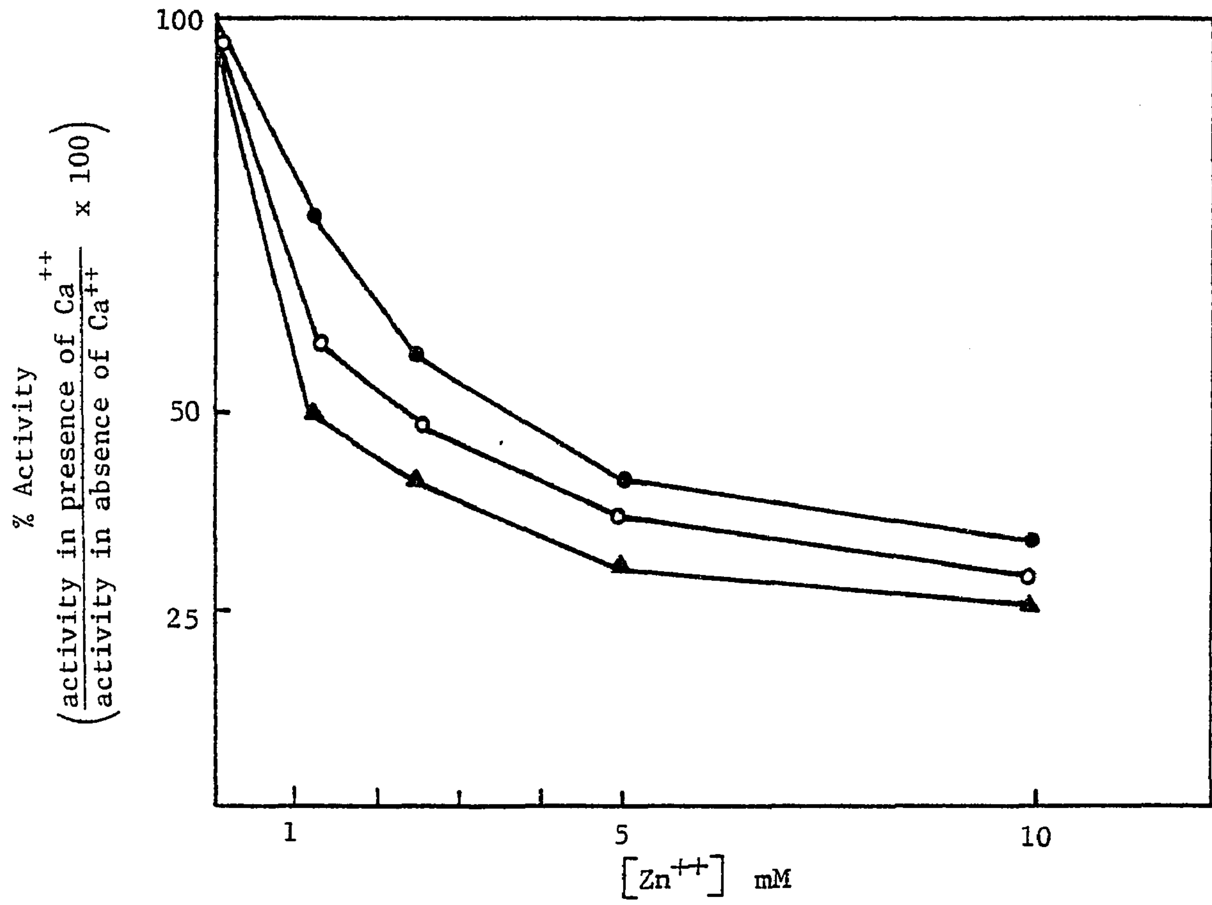


Fig. 15. The effect of Zn^{++} on protein kinase activity. The assay was carried out by the standard assay method described above in the presence of 1 μM cyclic AMP with the addition of $ZnCl_2$ to the final concentration from 0.5 to 10 mM. Histone was used as the substrate at a concentration of 1 mg/ml. [γ-^{32}P]-ATP was present at a concentration of 0.5 mM (50 cpm/pmol). The figure shows protein kinase activity in the presence of Zn^{++} expressed as a percentage of the protein kinase activity in the absence of Zn^{++} (control). The activity of cytosol protein kinase CPK II is indicated by •, membrane protein kinase MPK II by ∘, and unsolubilized membrane kinase by ▲.

Fig. 16(A)

Na^+

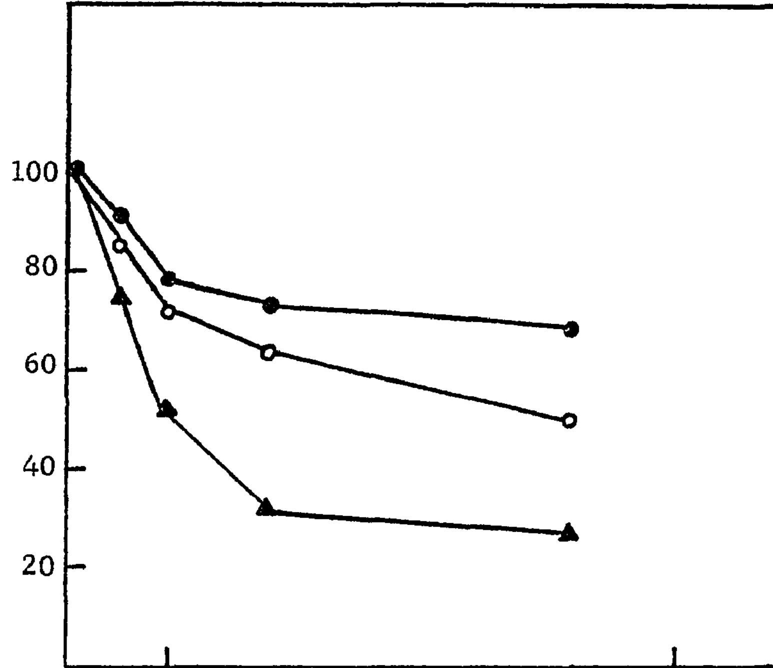


Fig. 16(B)

K^+

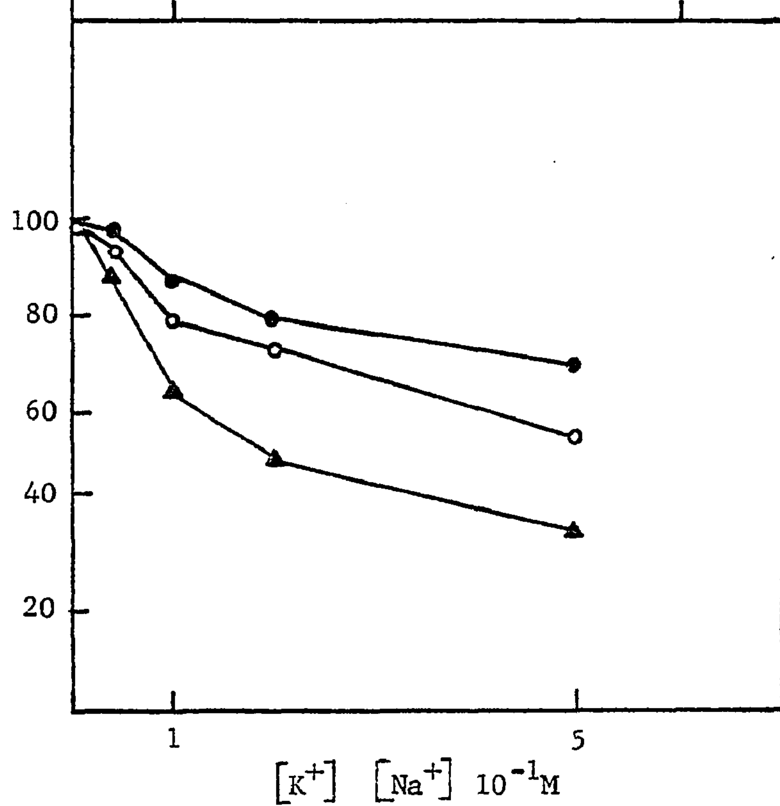


Fig. 16. The effect of K^+ and Na^+ on protein kinase activity. The assay was carried out by standard assay method described above in the presence of 1 μM with the addition of Na^+ (part A) or K^+ (part B) to the final concentration from 0.15 to 0.5 M. Histone was used as the substrate at a concentration of 1 mg/ml. [γ-^{32}P]-ATP was present at a concentration of 0.5 mM (50 cpm/pmol). The figure shows protein kinase activity in the present of Na^+ (part A) or K^+ (part B) as a percentage of the protein kinase activity in the absence of K^+ or Na^+ (control). The activity of cytosol protein kinase CPK II is indicated by •, membrane protein kinase MPK II by ∘, and unsolubilized membrane by ▲.

Fig. 17

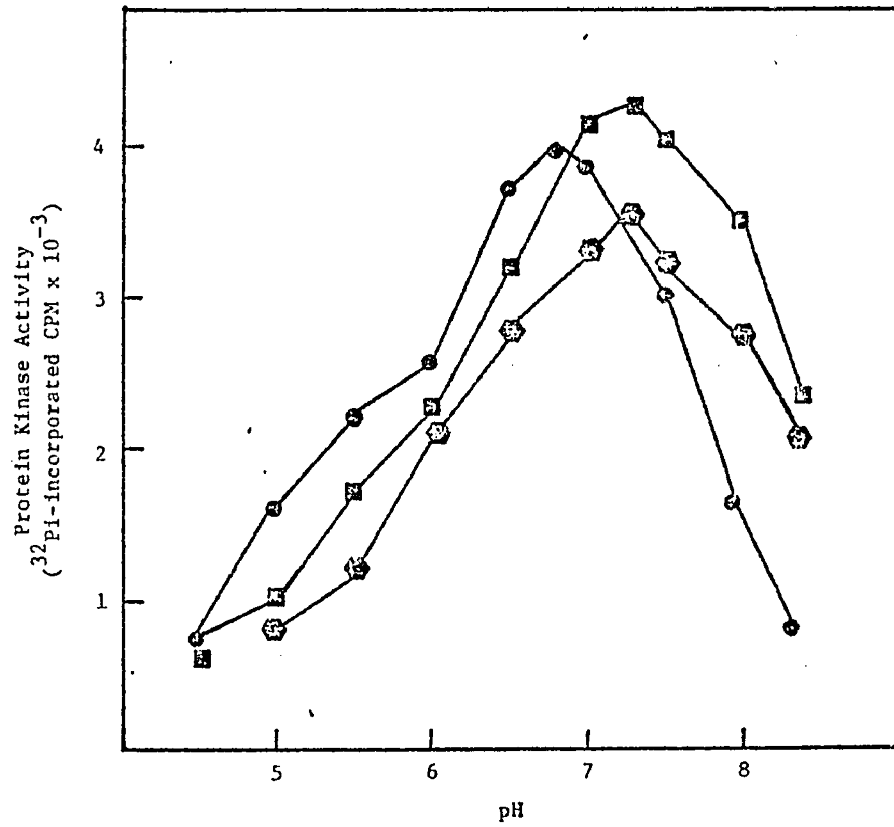


Fig. 17. pH effect on the activity of cyclic AMP-dependent protein kinase CPK I, CPK II and MPK II. The protein kinase was carried out with standard method, described in Experimental Procedure, but with the pH ranging from 4.5 to 8.5. The enzyme was assayed in the presence of 1 μM cyclic AMP. Histone was used as substrate at final concentration of 1 mg/ml, [γ-^{32}P]-ATP was presented at a concentration of 0.5 mM (50 cpm/pmol). The preparation was incubated for 10 min at 30°C. The following buffers at the indicated ranges of pH were used: 2-(N-morpholino)ethane sulfonate (MES) (4.5–5.5), imidazole (5.5–7.5), and Tris·HCl (7–8.5). The CPK I activity is indicated by •, CPK II, by ■, and MPK II, by ⬢.

Fig. 18

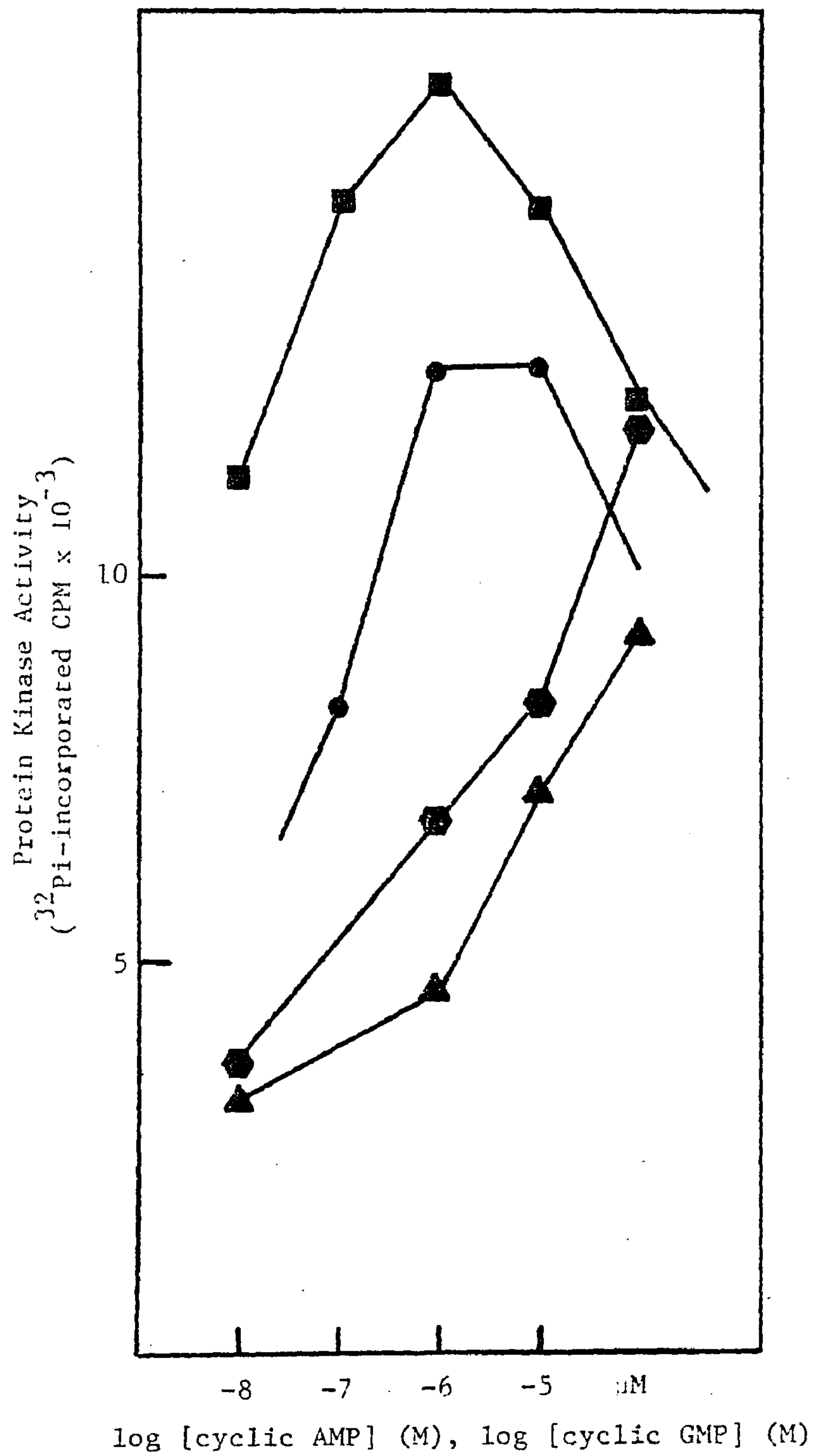


Fig. 18. The effect of concentration of cyclic AMP and cyclic GMP on protein kinase activity. The assay concentration of cyclic AMP and cyclic GMP ranged from 0.01 μM to 10 μM. The effect of cyclic AMP on protein kinase CPK II is indicated by ■ , and on protein kinase MPK II, by •. The effect of cyclic GMP on protein kinase CPK II is indicated by ⬢ , and on protein kinase MPK II, by ▲. Histone was used as substrate at final concentration of 0.5 mM, $[\gamma-^{32}P]$-ATP was present at a concentration of 0.5 mM (50 cpm/pmol). The enzyme concentration was 0.5 mg/ml, and the assay was carried out in accordance with the standard method described in the Experiment Procedure section, except that the cyclic AMP and cyclic GMP concentrations were changed.

Fig. 19

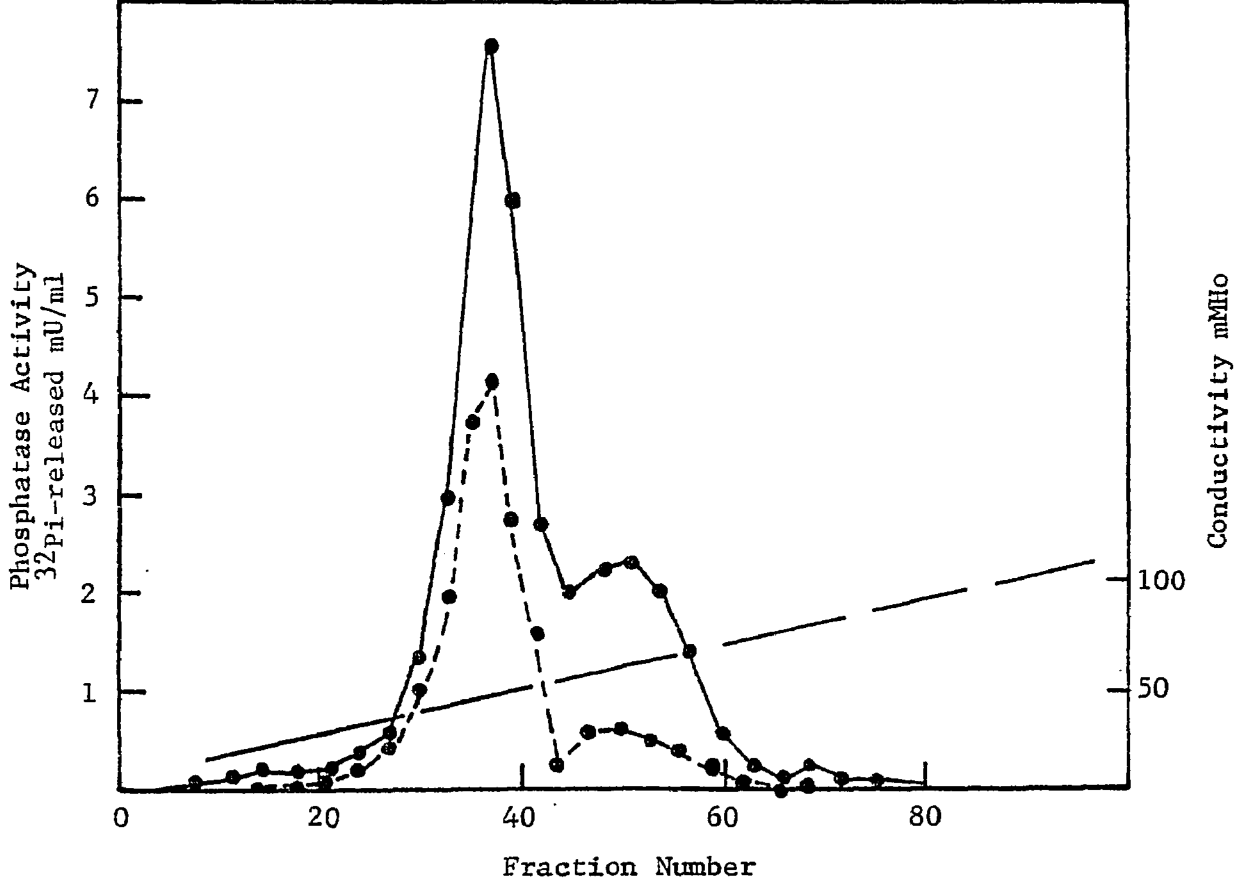


Fig. 19. Protein phosphatase activity of papillary plasma membrane fraction separated on DEAE-Sepharose CL-6B column chromatography. The solubilized plasma membrane fraction (22 ml, 7.5 mg/ml) containing protein phosphatase was applied to a DEAE-Sepharose CL-6B column (1 x 30 cm). After the sample was applied, the column was eluted with 500 ml of a linear gradient (50-400 mM) of KCl. One hundred and four fractions were collected at the volume of 4.9 ml per fraction. The histone and casein phosphatase activity was measured using a standard assay mixture containing 50 mM Tris•HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MnCl_2$, 0.1 M KCl, and 10 μM ^{32}P-labelled substrate protein and 10 μl of eluted enzyme in a volume of 50 μl for 10 min at 30°C. The histone phosphatase is indicated by —o—, and casein phosphatase, by --o--.

Fig. 20

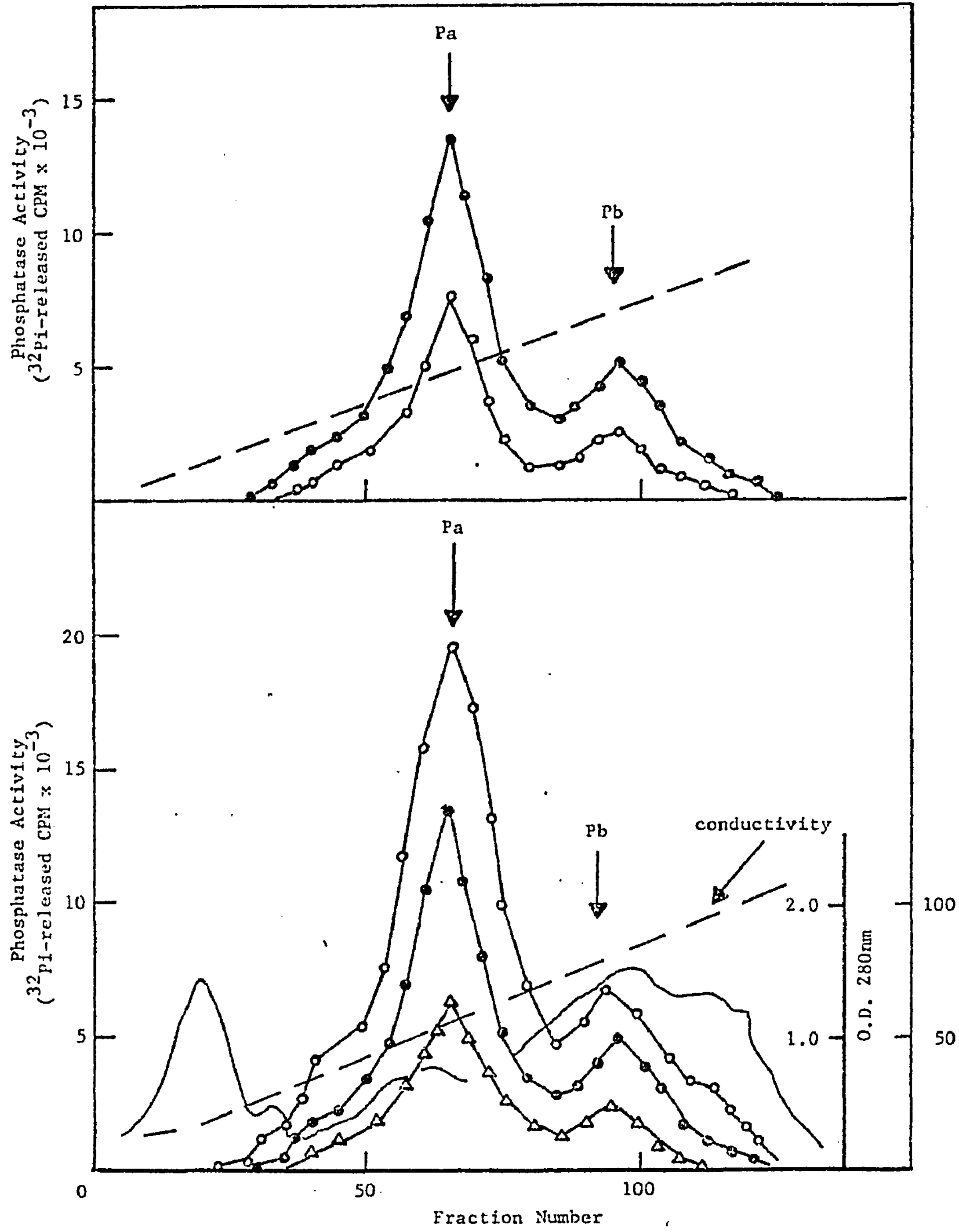


Fig. 20a. <u>Protein phosphatase activity of the cytosol fraction separated on DEAE-cellulose column chromatography.</u> The cytosol fraction precipitated by 55% $(NH_4)_2SO$ (20 ml, 12 mg/ml) was applied to a DEAE-cellulose (Whatman DE-52) column (1.5 x 40 cm). The enzyme was eluted with 500 ml of a gradient (20-400 mM) of buffer A-KCl. One hundred twenty-five fractions were collected each with volume of 4.1 ml. The figure shows the elution profile of protein phosphatase activity assayed on 10 µl aliquots of the indicated fraction by the standard method described in the Experimental Procedure section. The histone phosphatase activity is indicated by •, and casein phosphatase activity, by ○.

Fig. 20b. <u>The effect of Mn^{++} and ATP on the cytosol protein phosphatase</u>. The figure shows the same elution as in Fig. 20a. The assay was carried out by the standard method described above with the addition of 5 mM $MnCl_2$ or 2 mM ATP. [^{32}P]-histone was used as the substrate. The figure shows the elution profile of protein phosphatase activity assayed on 10 μl aliquots of the indicated fractions. The protein phosphatase activity is indicated by • in the absence of $MnCl_2$ and ATP. In the presence of 5 mM $MnCl_2$ the phosphatase activity is indicated by ∘, and in the presence of 2 mM ATP, by Δ.

Fig. 21

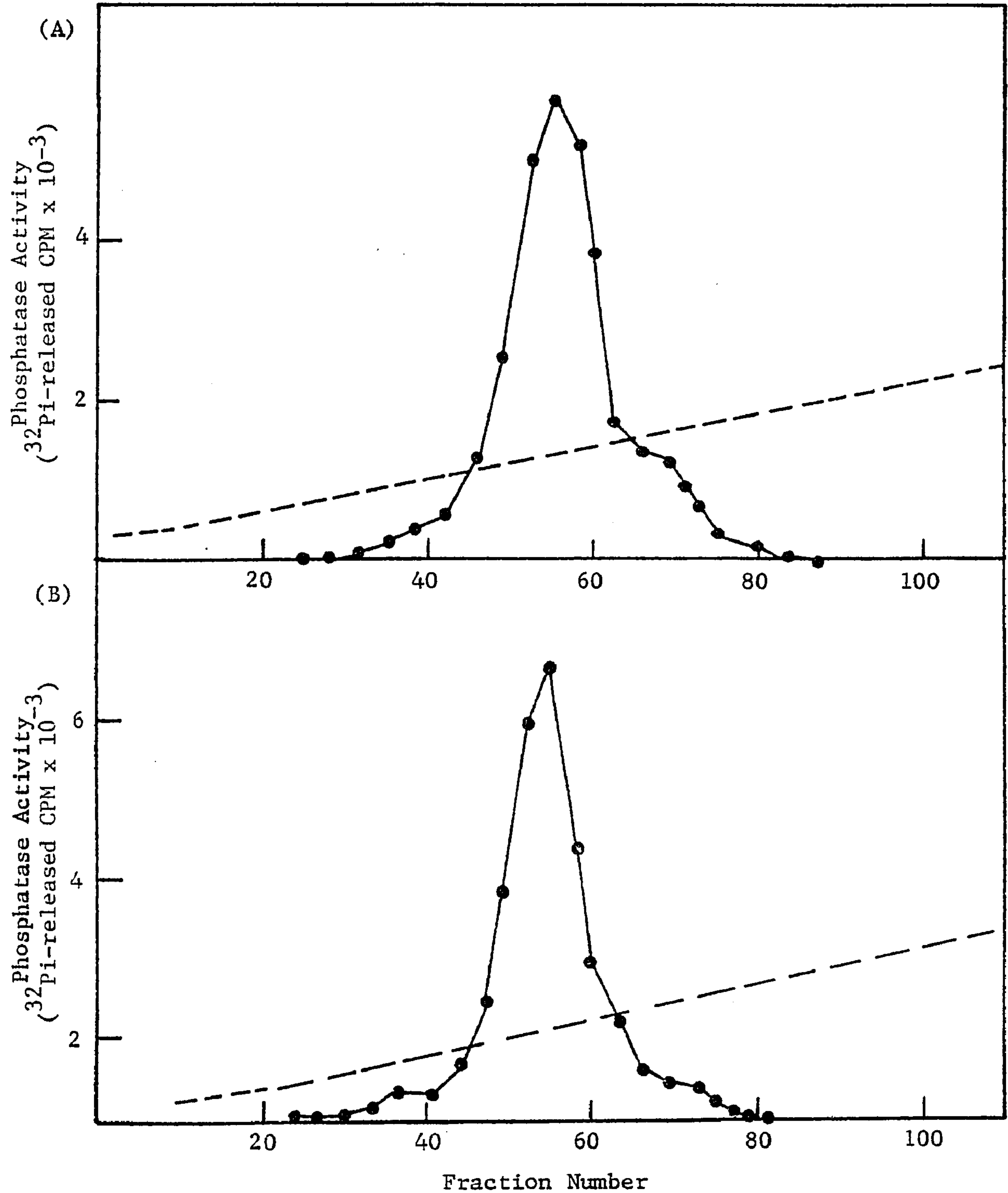


Fig. 21a. DEAE-cellulose chromatography of ethanol-treated renal cytosol protein phosphatase Pa and Pb. The cytosol protein phosphatase Pa and Pb were eluted from a DEAE-cellulose (Whatman DE-52) column (1.2 x 30 cm) pre-equilibrated with buffer A, pooled and treated with ethanol with a procedure described in the Experimental Procedure section. Ten ml (5 mg/ml) of treated enzyme concentrate was rechromatographed on the same size of DEAE-cellulose column and eluted with 400 ml of a gradient (20-400 mM) of buffer A-KCl. One hundred eight fractions were collected each with volume of 3.8 ml. The protein phosphatase assay was carried out by the standard method described above. [^{32}P]-histone was used as the substrate. The figure shows the elution profile of histone phosphatase activity assayed on 10 μl aliquots of the indicated fractions. The histone phosphatase activity is indicated by •, and conductivity by ---.

Fig. 21b. <u>DEAE-cellulose chromatography of renal cytosol protein phosphatase Pa with ethanol-treated protein phosphatase Pb</u>. Renal cytosol protein phosphatase Pa combined with ethanol-treated protein phosphatase Pb was eluted from a DEAE-cellulose (Whatman DE-52) column (1.2 x 30 cm). Twelve ml (6 mg/ml) of enzyme was rechromatographed on the same size of DEAE-cellulose DE-52 column pre-equilibrated with buffer A and eluted with 400 ml of a gradient (20-400 mM) of buffer A-KCl. One hundred eighteen fractions were collected each with volume of 3.7 ml. The protein phosphatase assay was carried out by the standard method described above. [^{32}P]-histone was used as the substrate. The figure shows the elution profile of histone phosphatase activity assayed on 10 μl aliquots of the indicated fractions. The histone phosphatase activity is indicated by • and conductivity by ---.

Fig. 22

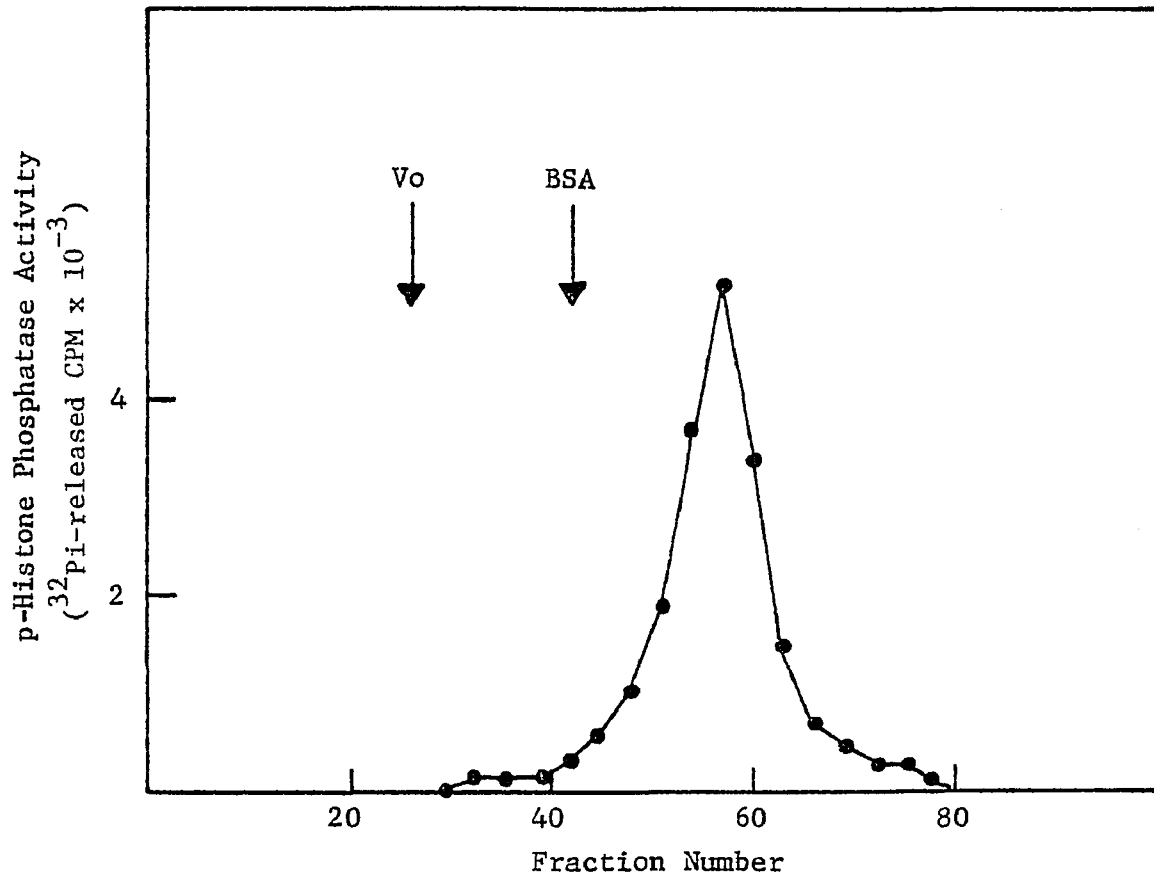


Fig. 22. Sephadex G-100 chromatography of the ethanol-treated renal cytosol protein phosphatase Pb fraction and cytosolic protein phosphatase Pa fraction. Four ml (7.6 mg/ml) of the combined enzyme fraction was applied to a Sephadex G-100 column (2 x 45 cm) which was pre-equilibrated with buffer A. The enzyme was eluted with the same buffer. Fractions with a volume of 3.2 ml were collected. Aliquots (10 µl) of the indicated fractions were assayed for histone phosphatase activity by the standard method described in the Experimental Procedure section; the preparation was incubated for 10 min at 30°C. V_o refers to the void volume. BSA indicates the elution volume of bovine serum albumin.

Fig. 23(A)

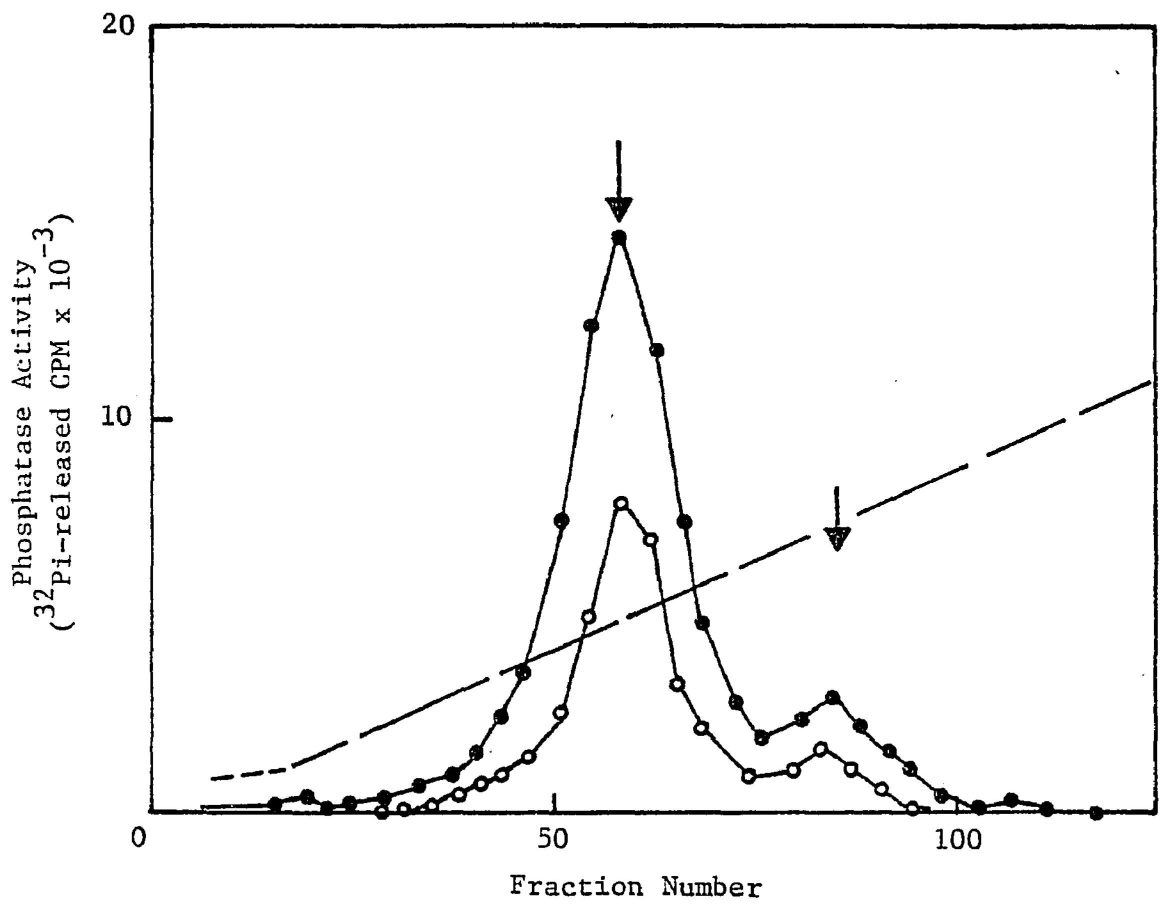


Fig. 23(B)

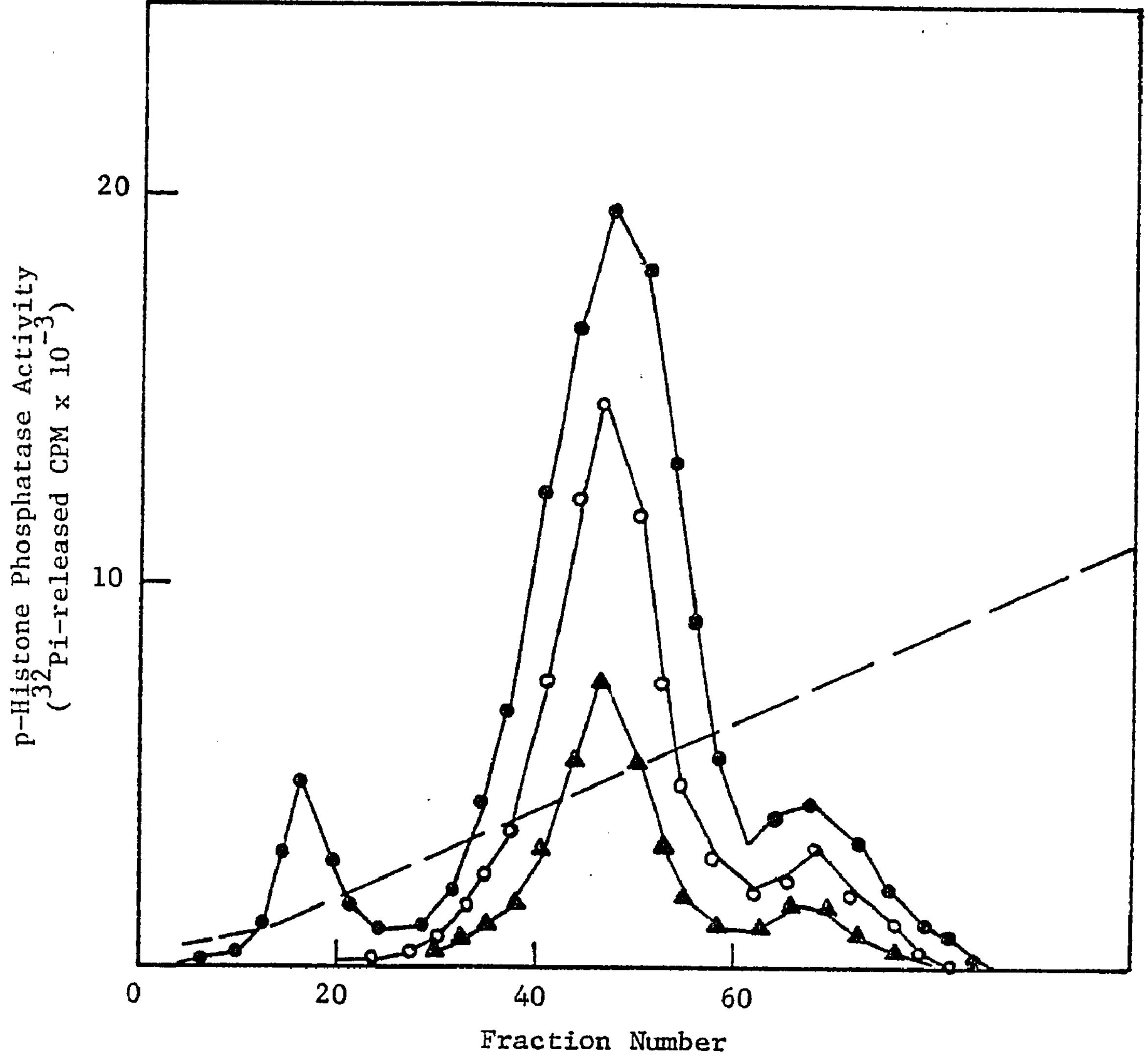


Fig. 23. Phosphatase activity of the cytosol fraction of renal cortex separated on DEAE-cellulose column chromatography. The renal cortex cytosol fraction precipitated by 55% $(NH_4)_2SO$ (26 ml, 9.5 mg/ml) was applied to a column of DEAE-cellulose (Whatman DE-52) (2.6 x 52 cm). The enzyme was eluted with 600 ml of a gradient (20-400 mM) of buffer A-KCl (pH 7.0). One hundred twenty one fractions were collected each with a volume of 5.2 ml. The enzyme activity was assayed on 10 µl aliquots of the indicated eluted fractions either with or without 5 mM $MnCl_2$ or 2mM ATP by the standard method described in the Experimental Procedure section. $[^{32}P]$-Histone and $[^{32}P]$-casein were used as substrate. The incubation was carried out for 10 min at 30°C. Part A shows the protein phosphatase activity using $[^{32}P]$-histone (•) and $[^{32}P]$-casein (○) as substrates. Part B shows the elution profile of histone phosphatase activity assayed in the presence of 5 mM $MnCl_2$ (•) and in the presence of 2 mM ATP (▲) using $[^{32}P]$-histone as substrate.

Fig. 24

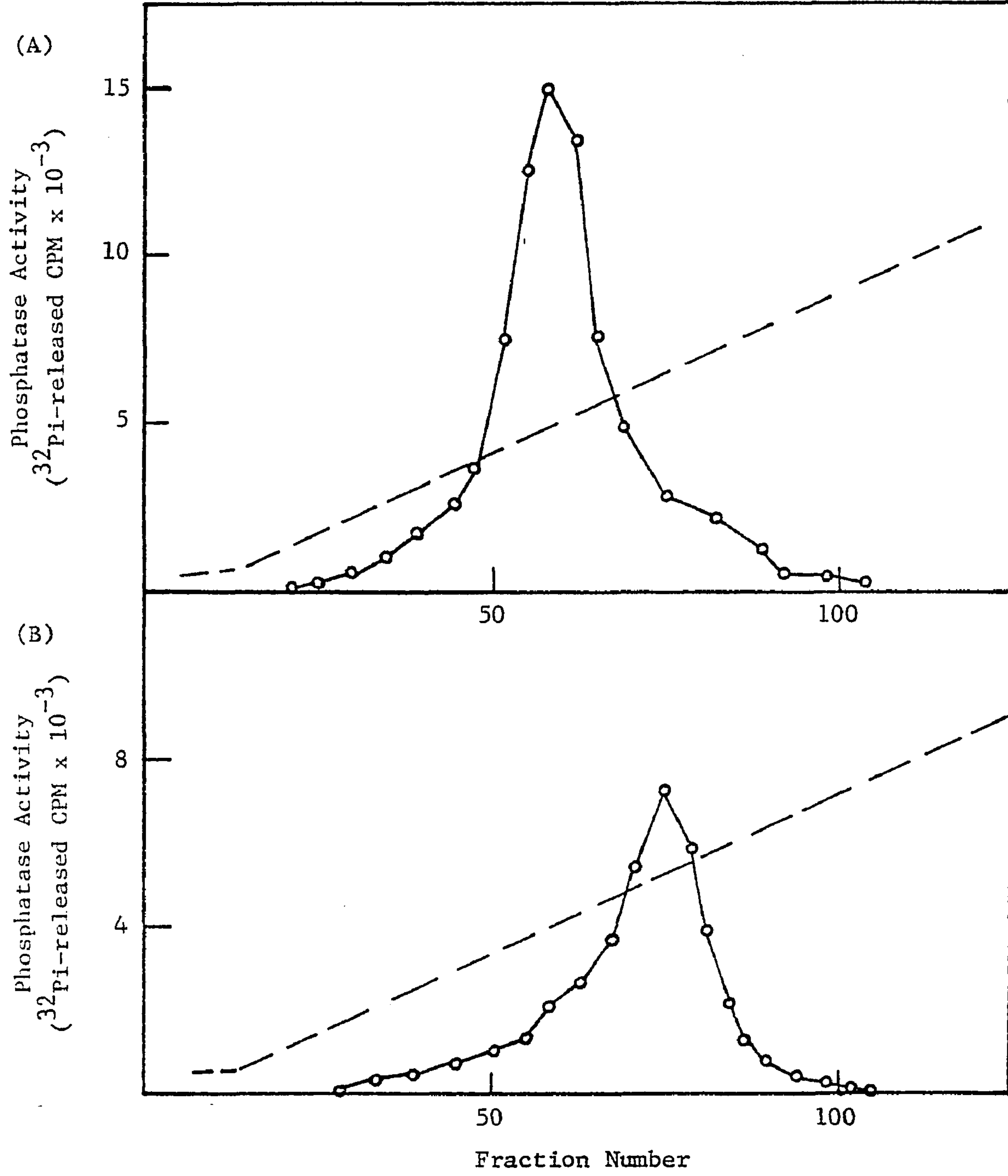


Fig. 24a. DEAE-cellulose chromatography of the major peak of renal cortical phosphatase combined with the major peak of medullary phosphatase. The combined enzyme (12 ml, 12 mg/ml) was applied to a DEAE-cellulose column (1.5 x 45 cm). The enzyme was eluted with 400 ml of a gradient (20-400 mM) of buffer A-KCl (pH 7.0) as in the method described above; one hundred ten fractions of 3.7 ml were collected. The figure shows the elution profile of histone phosphatase (○). A 10 µl aliquot from each of the indicated fractions was assayed by the standard method described above. The incubation was carried out for 10 min at 30°C.

Fig. 24b. DEAE-cellulose chromatography of the minor peak of cortical phosphatase combined with renal medullary phosphatase Pb. The combined enzyme (6 ml, 6.7 mg/ml) was applied to a DEAE-cellulose (Whatman DE-52) column (1.5 x 45 cm) pre-equilibrated with buffer A. The enzyme was eluted with 400 ml of a gradient (20-400 mM) of buffer A-KCl (pH 7.0). A volume of 3.6 ml was collected for each fraction. A 10 μl aliquot from each of the indicated fractions was assayed for histone phosphatase activity. The assay was carried out by the standard assay method described above. The incubation was carried out for 10 min at 30°C. The histone phosphatase activity is indicated by $^{\circ}$ and conductivity by ---.

Fig. 25

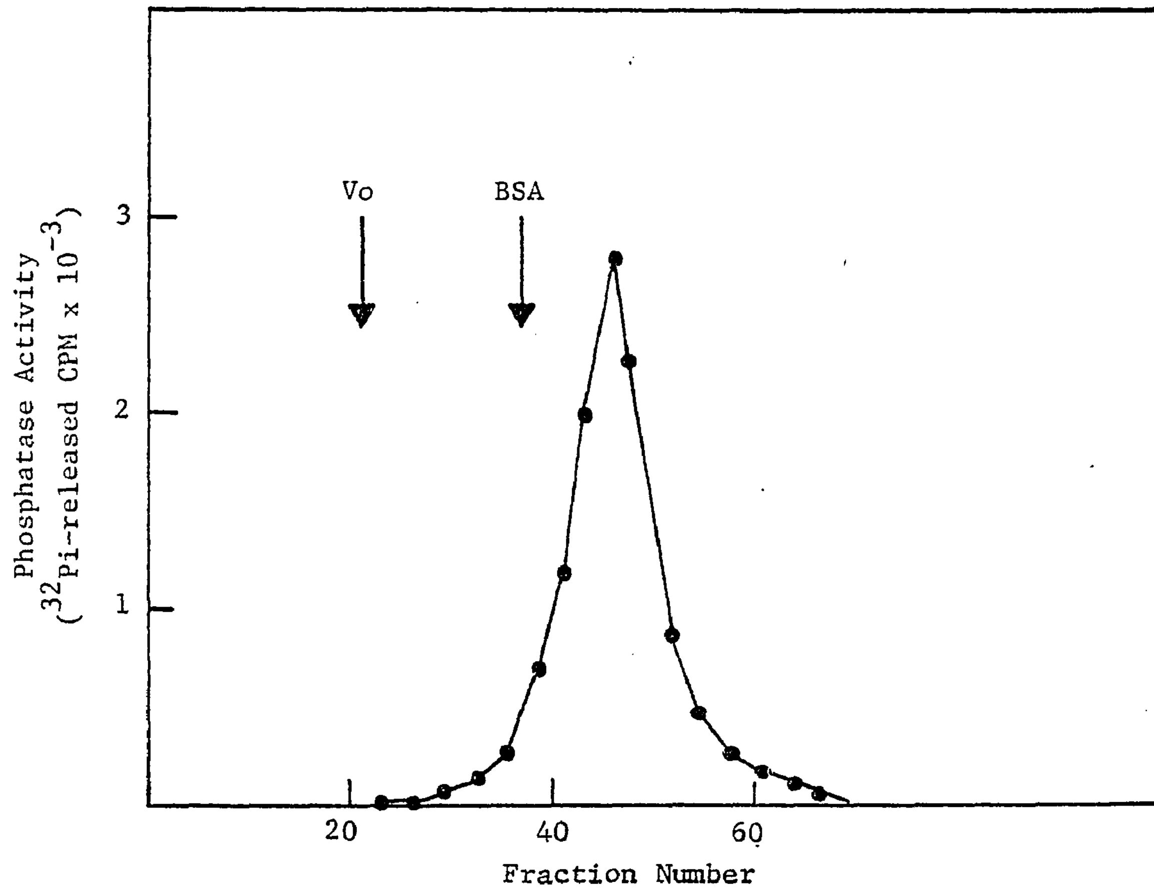
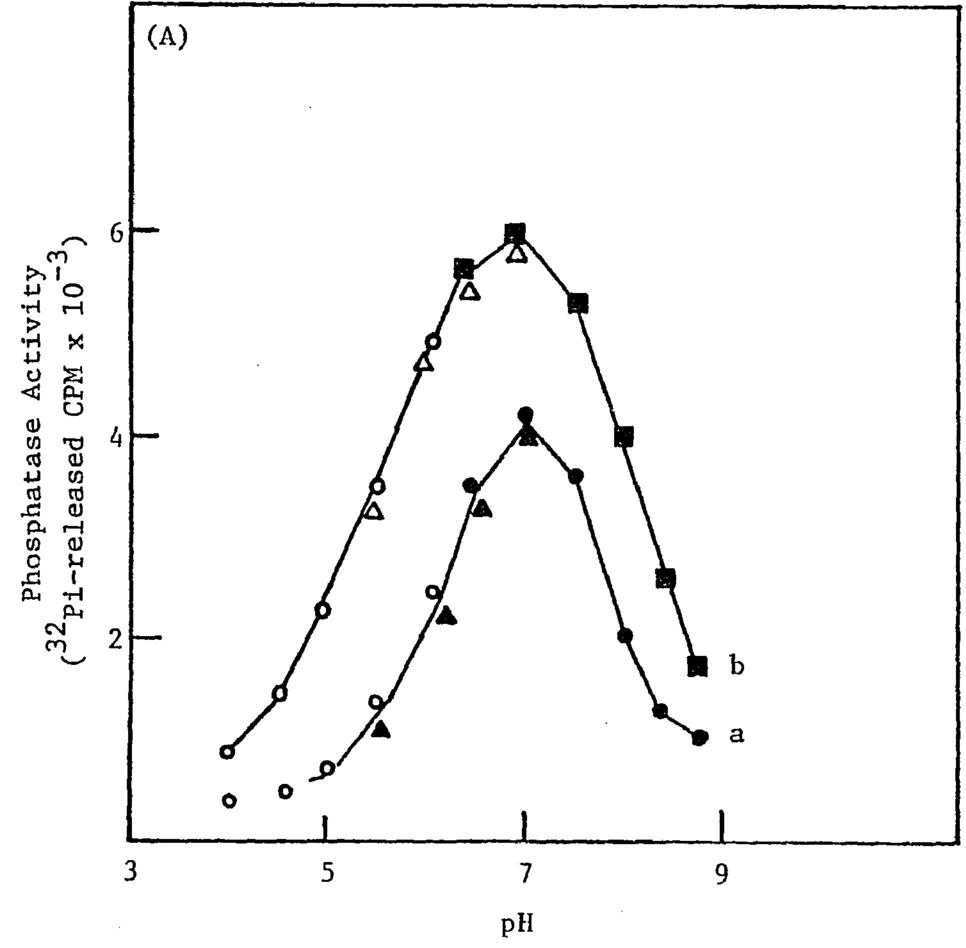


Fig. 25. Sephadex G-100 chromatography of the major peak of renal cortical cytosol phosphatase combined with the major peak of renal medullary cytosol phosphatase Pa. The combined enzyme 5 ml (8 mg/ml) was applied on a Sephadex G-100 column (2.5 x 58 cm) pre-equilibrated with buffer A. The enzyme was also eluted with buffer A. A volume of 4.1 ml was collected for each fraction. A 10 µl aliquot from each of the indicated fractions was assayed for histone phosphatase activity by the standard method described above. V_o refers to the void volume. BSA indicates the elution volume of bovine serum albumin.

Fig. 26



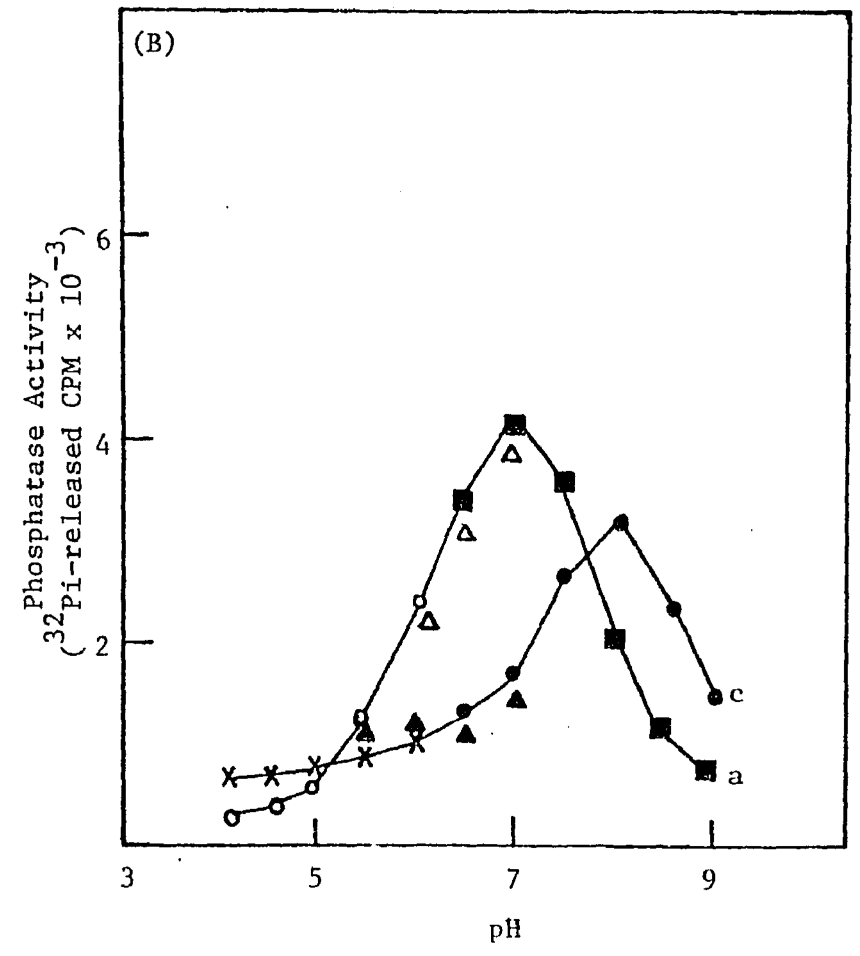


Fig. 26. Effect of pH on the activity of cytosol phosphatase Pa. The enzyme activity was measured in an incubation volume of 50 μl containing 50 mM buffer, 1 mM dithiothreitol, 0.1 M KCl, 10 μg of phosphatase Pa and 10 μM [^{32}P]-histone. Incubation was carried out for 10 min at 30°C. The following buffers at the indicated ranges of pH were used: 2-(N-morpholiono)ethane sulfonate (MES) (4.5-5.5), imidazole (5.5-7.5), Tris•HCl (7.0-8.5). Part A shows the phosphatase activity in the absence of $MnCl_2$ (a) and in the presence of 5 mM $MnCl_2$ (b). Part B shows the phosphatase activity in the absence of ATP (a) and in the presence of 2 mM ATP (c). The assay was carried out by the standard method described above.

Fig. 27

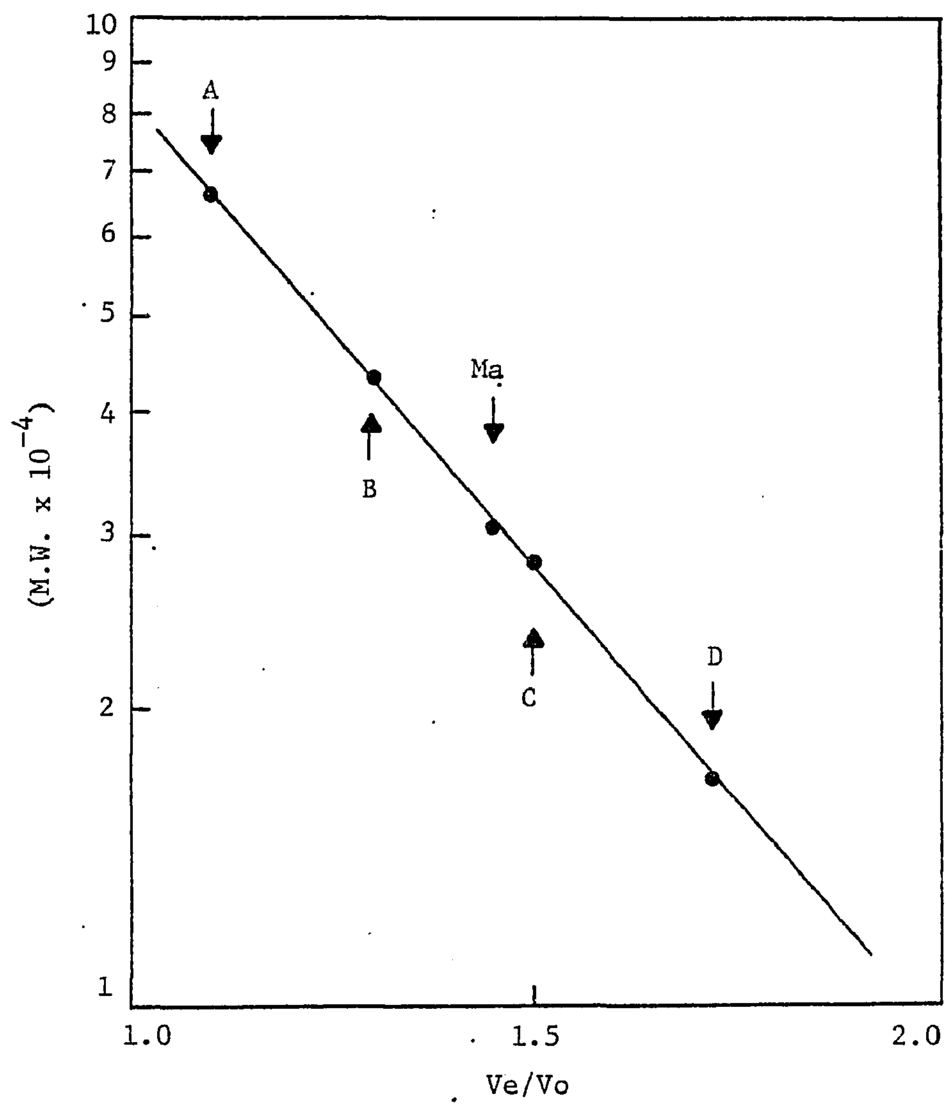


Fig. 27. Determination of the molecular weight of membrane-bound protein phosphatase Ma by Sephadex G-100 gel filtration. The molecular weight of phosphatase Ma was determined by gel filtration on a Sephadex G-100 column (1.6 x 88 cm). The following proteins were used as standards for calibration:

A bovine serum albumin (Mr = 67,000)

B ovalbumin (Mr = 43,000)

C carbonic anhydrase (Mr = 29,000)

D myoglobin (Mr = 17,000)

V_o indicates the void volume

Fig. 28

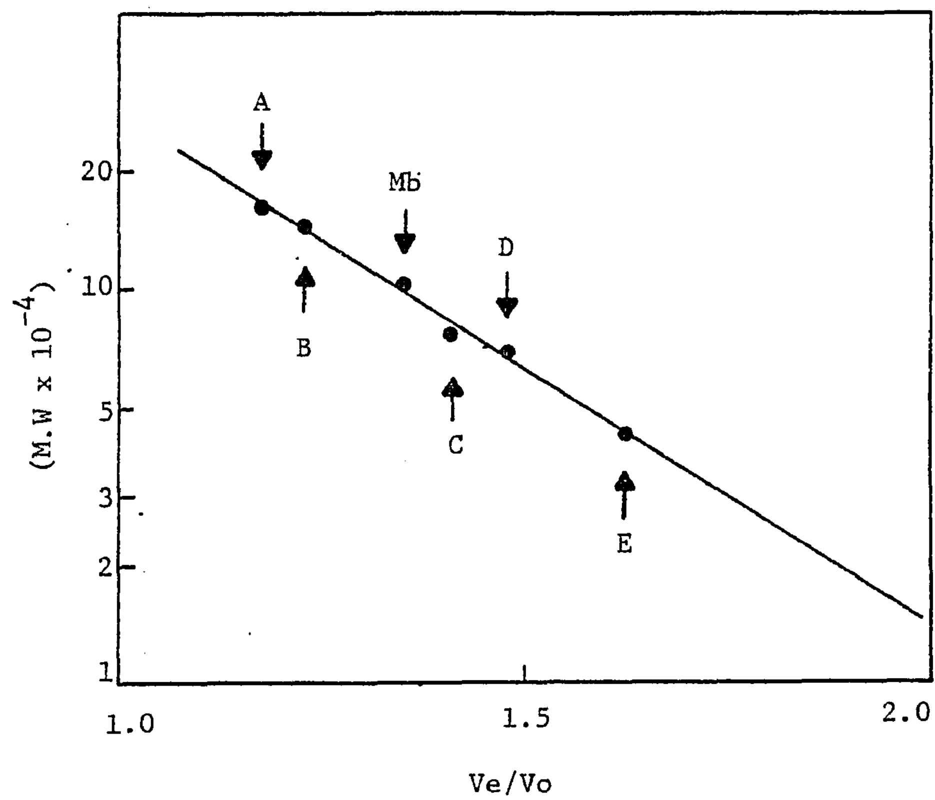


Fig. 28. Determination of the molecular weight of membrane-bound protein phosphatase Mb by Sephadex G-200 gel filtration. The molecular weight of phosphatase Mb was determined by gel filtration on a Sephadex G-200 column (2.5 x 60 cm). The following proteins were used as standards:

A γ-globulin (Mr = 167,000)

B lactate dehydrogenase (Mr = 140,000)

C alcohol dehydrogenase (horse liver) (Mr = 80,000)

D bovine serum albumin (Mr = 67,000)

E ovalbumin (Mr = 43,000)

Fig. 29

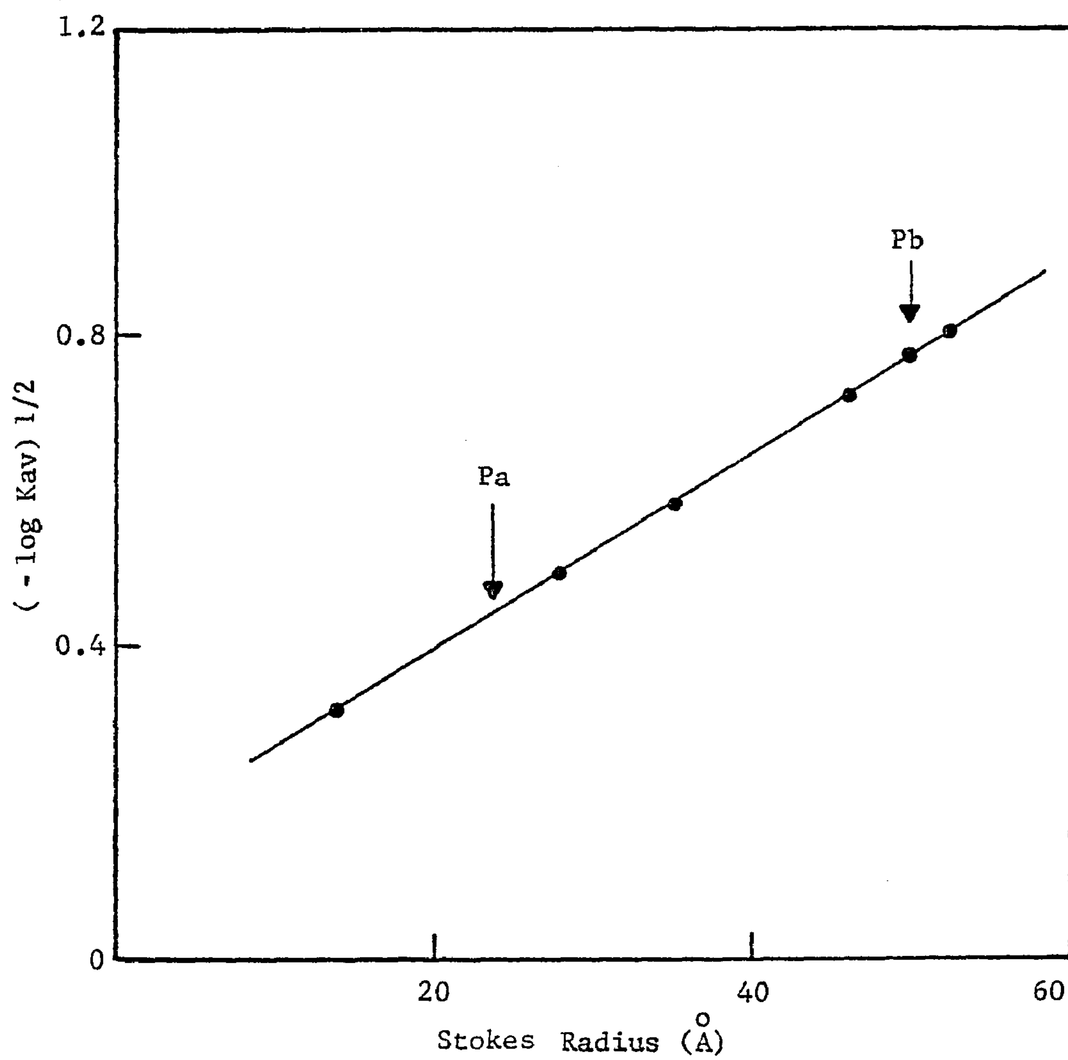


Fig. 29. Determination of the Stokes radii of phosphatases Pa and Pb by Sephadex G-200 gel filtration. Phosphatases Pa and Pb were eluted from Sephadex G-200, and the column was calibrated with the following proteins (a = Stokes radius): bovine γ-globulin (a = 52 Å), yeast alcohol dehydrogenase (a = 46 Å), bovine serum albumin (a = 35 Å), ovalbumin (a = 28 Å), cytochrome *c* (a = 17 Å). The data are plotted according to the method of Siegel and Monty (251).

Fig. 30

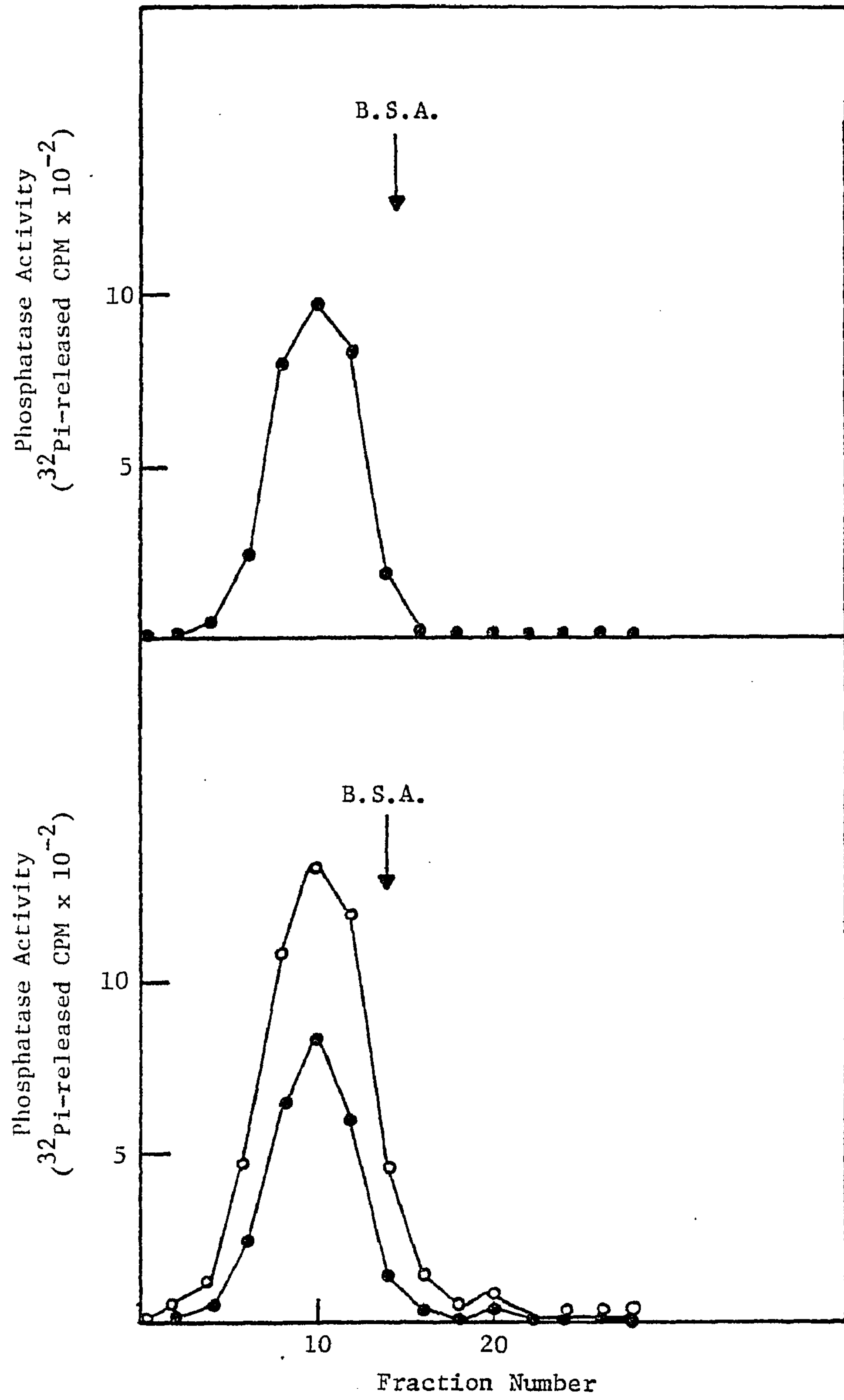


Fig. 30. Sucrose density gradient ultracentrifugation of protein phosphatases Pa and Pb. Samples of 0.1 ml approximately 3 mg/ml were layered onto sucrose gradient (5-20% in 20 mM Tris, pH 7.4) and centrifuged in an SW 50.1 rotor at 39,000 rpm for 18 hr at 4°C. Fractions of 3 drops were collected from the bottom of the tubes. Histone phosphatase activity was measured in an incubation volume of 100 μl containing 50 mM Tris•HCl (pH 7.4), 1 mM dithiothreitol, 0.1 m KCl, and 10 μM [^{32}P]-histone with or without 5 mM $MnCl_2$. The incubation was carried out for 10 min at 30°C. The enzymatic activity was expressed in counts/min. In part A, the protein phosphatse Ma activity is indicated by •, and in part B, the protein phosphatase Pa activity is indicated by ∘ in the presence of 5 mM $MnCl_2$ and by • in the absence of $MnCl_2$. BSA designates bovine serum albumin.

Fig. 31

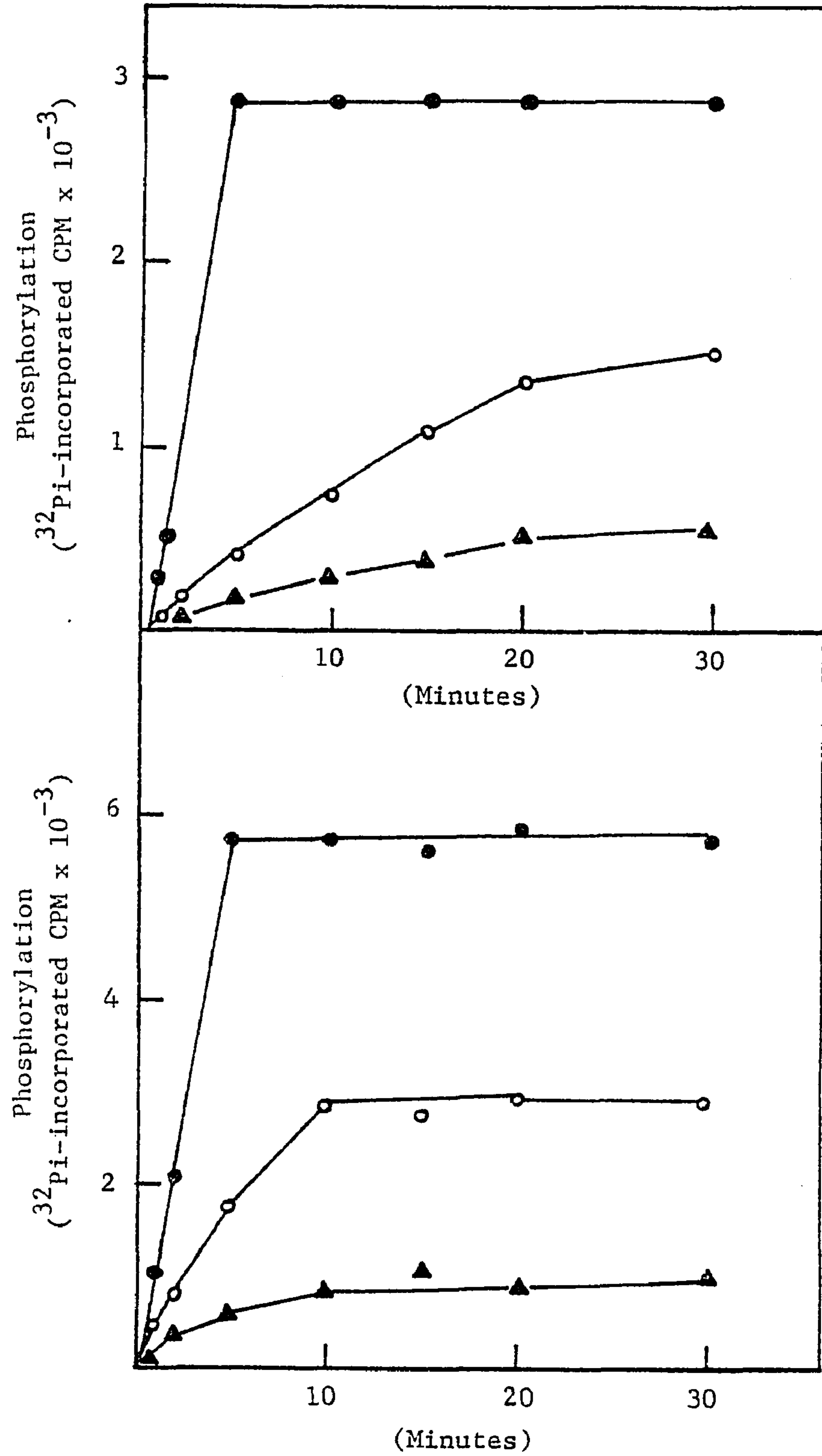


Fig. 31. The time course of the phosphorylation of papillary plasma membrane. The phosphorylation assay was carried out in a reaction mixture of 100 μl containing 50 mM KPi (pH 7.0), 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]-ATP (100 cpm/pmol), 10 mM NaF, 1 mM theophylline, and with or without the presence of 5 μM cyclic AMP or with the addition of 10 μg purified protein kinase catalytic subunit (bovine heart), and unsolubilized (part A) 100 μg or solubilized (part B) plasma membrane 150 μg. The incubation time ranged from one to thirty minutes at room temperature. In both parts, the phosphorylation of membrane in the absence of cyclic AMP is indicated by ▲, in the presence of cyclic AMP by ∘, and upon the addition of the catalytic subunit of protein kinase by •.

Fig. 32

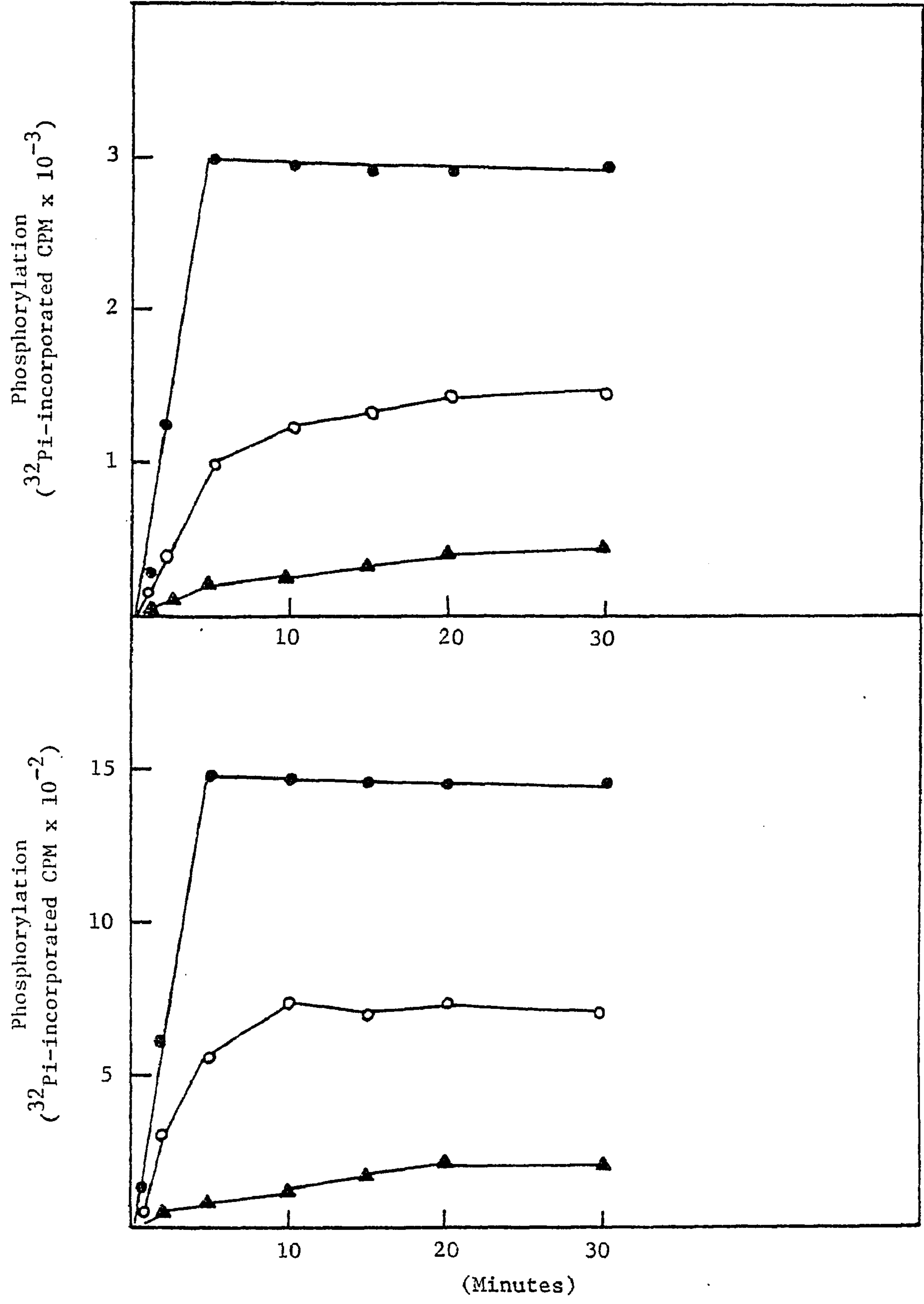


Fig. 32. The time course of the phosphorylation of histone and plasma membrane by protein kinases CPK II and MPK II. The phosphorylation was carried out by the standard kinase assay method described above in the reaction mixture with volume of 100 μl containing 50 mM KPi (pH 7.0), 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (100 cpm/pmol), 10 mM NaF, 5 μM cyclic AMP, 50 μg to 100 μg of substrate and 20 μg of enzyme. The substrates were histone, solubilized membrane, and unsolubilized membrane. The enzymes were protein kinase CPK II (part A) and MPK II (part B). The incubation time ranged from one to thirty minutes. In both parts, the phosphorylation of histone is indicated by •, the phosphorylation of solubilized plasma membrane, by ∘, and the phosphorylation of unsolubilized membrane, by ▲.

Fig. 33

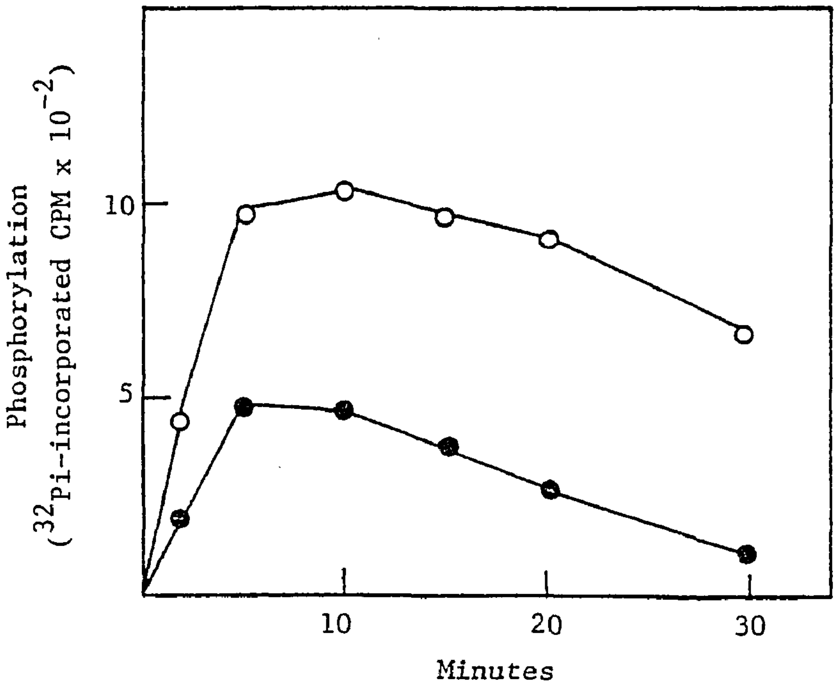


Fig. 34

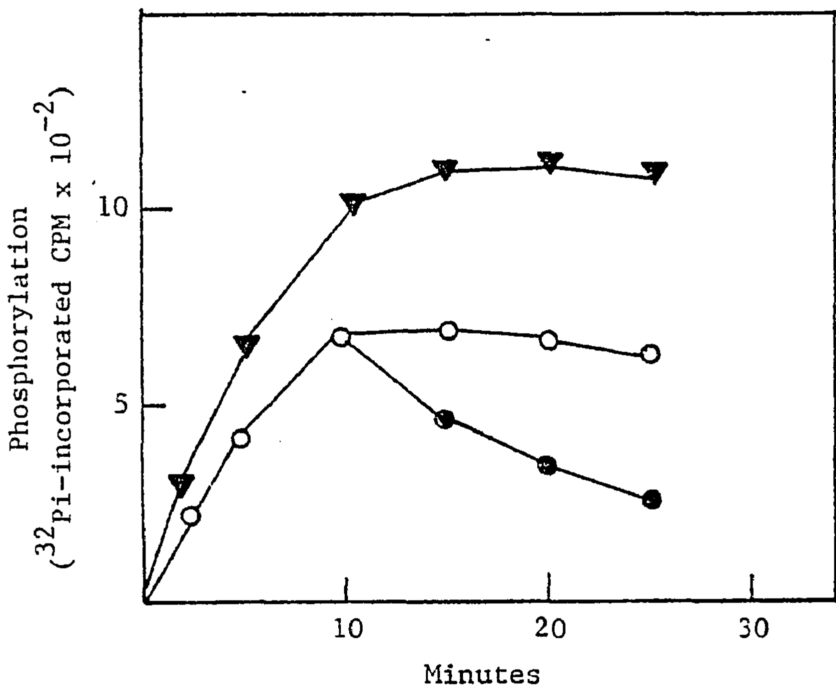


Fig. 33. The effect of membrane-bound phosphatase on the phosphorylation of plasma membrane. The phosphorylation of solubilized plasma membrane was carried out in the absence of protein phosphatase inhibitor by the standard method described above. The reaction mixture was in a volume of 100 μl containing 50 μM KPi (pH 7.0), 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (50 cpm/pmol), 1 mM theophylline, 100 μg solubilized plasma membrane and with 5 μM cyclic AMP or in the absence of cyclic AMP but with 20 μg protein kinase catalytic subunit purified from bovine heart. The incubation time range from two to thirty min at room temperature. The phosphorylation of plasma membrane with the addition of protein kinase catalytic subunit in the absence of cyclic AMP is indicated by ∘ and by • in the presence of 5 μM cyclic AMP.

Fig. 34. The effect of NaF and Zn^{++} on the endogenous phosphorylation of plasma membrane. The endogenous phosphorylation of solubilized membrane was carried out in a reaction mixture of 100 µl containing 50 µM KPi (pH 7.0), 100 mM $MgCl_2$, 0.5 mM [γ-^{32}P]-ATP (50 cpm/pmol), 1 mM theophylline, 100 µg solubilized plasma membrane, 5 µM cyclic AMP and with 20 mM NaF or 2 mM Zn^{++} or in the presence of 2 mM Zn^{++} upon the addition of 20 mM EDTA at time 10 min. The incubation time range from two to thirty minutes. The assay in the presence of 20 mM NaF is indicated by ▼, in the presence of 2 mM Zn^{++} by ∘, and in the presence of 2 mM Zn^{++} upon the addition of 20 mM EDTA at time 10 min by •.

Fig. 35

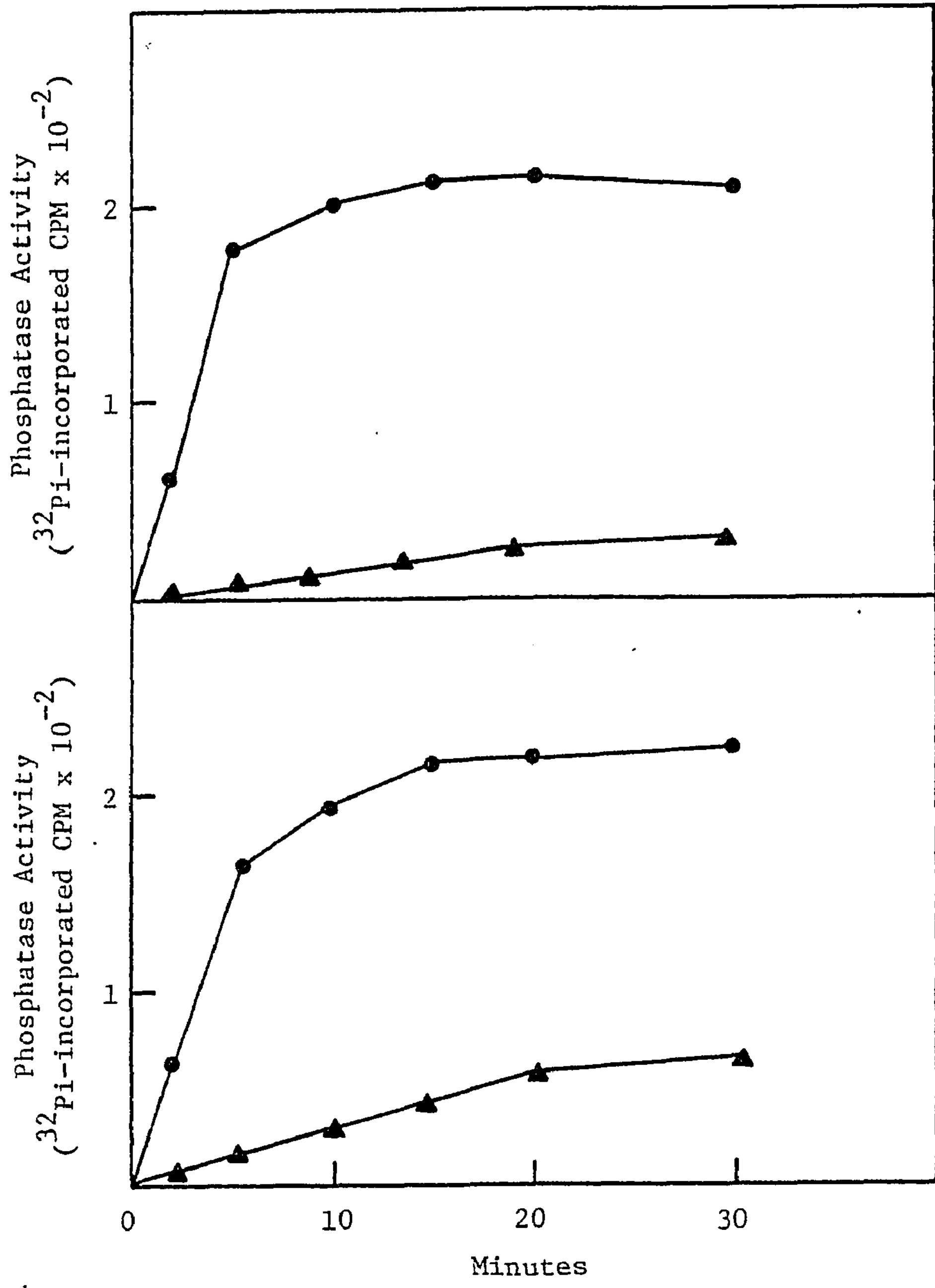


Fig. 35. The time course of dephosphorylation of phosphorylated plasma membrane and histone by protein phosphatases Pa and Ma. The dephosphorylation of plasma membrane was carried out by standard assay described above. The reaction mixture was in a volume of 50 μl containing 50 mM Tris·HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MnCl_2$, 0.1 M KCl, 20 μg of $[^{32}P]$-histone or approximately 50 μg of ^{32}P label phosphorylated membrane which was prepared by the method described in the Experimental Procedure section, 10 μg of enzyme (phosphatase Pa and Ma). In part A the enzyme was cytosol phosphatase Pa and in part B was membrane phosphatase Ma. In both parts, the dephosphorylation of histone is indicated by •, and dephosphorylation of plasma membrane by ▲.

Fig. 36

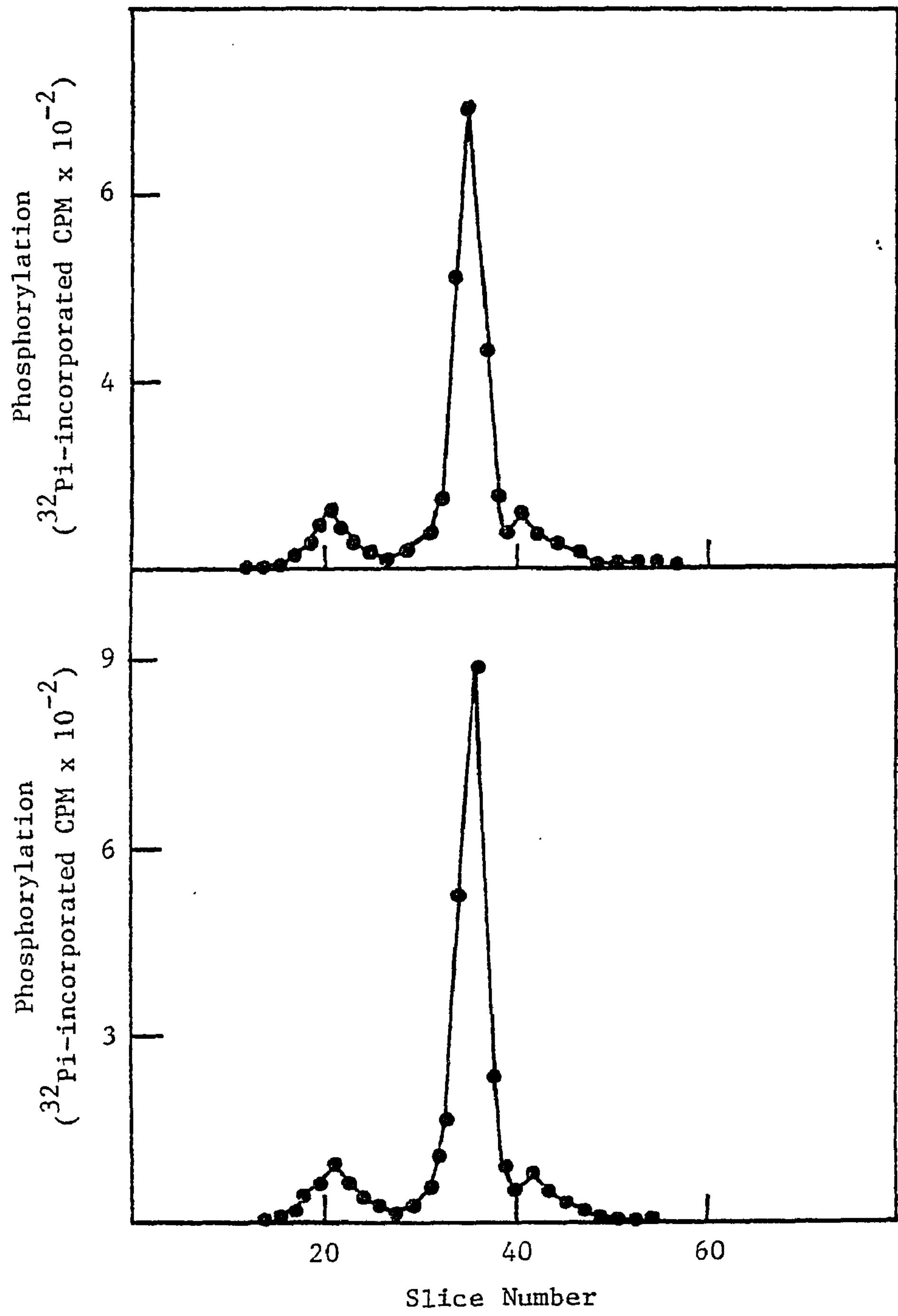


Fig. 36. SDS gel electrophoresis of phosphorylated plasma membrane. The assay was carried out in a final volume of 100 μl in a mixture containing 50 mM Tris (pH 7.0), 10 mM $MgCl_2$, 50 mM [γ-^{32}P] ATP (200 cpm/pmol), with or without 5 μM cyclic AMP, 250 μg of 1% Triton X-100 solubilized plasma membrane. Incubation was performed for 10 min at 30°C. The reaction was stopped by adding 1 ml of ice cold 40% trichloroacetic acid. The assay tubes were centrifuged in a table centrifuge for 1 min. The supernatants were discarded, same volume of water added and the tubes recentrifuged. The pellets were suspended in 50 μl of SDS solubilizing solution, which was comprised of 2% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol, 20 mM sodium phosphate (pH 7.0). After the sample was solubilized, 80 μl of solution B, which was comprised of 0.1 mM EDTA, 1% 2-mercaptoethanol, 20 mM sodium phosphate (pH 7.0), 40% sucrose and 0.005% bromide blue was added to the sample. Then 50 μl of the final solution was subjected to SDS gel electrophoresis. The electrophoresis was performed according to the procedure of Laemmli (207) employing a 10% polyacrylamide gel. The gel was sliced into 1 mm sections. The sliced sections were placed in toluene scintillation fluid and counted. Part A shows the phosphorylation in the absence of cyclic AMP, and part B shows the phosphorylation in the presence of 5 μM cyclic AMP and 20 mM NaF.

Fig. 37

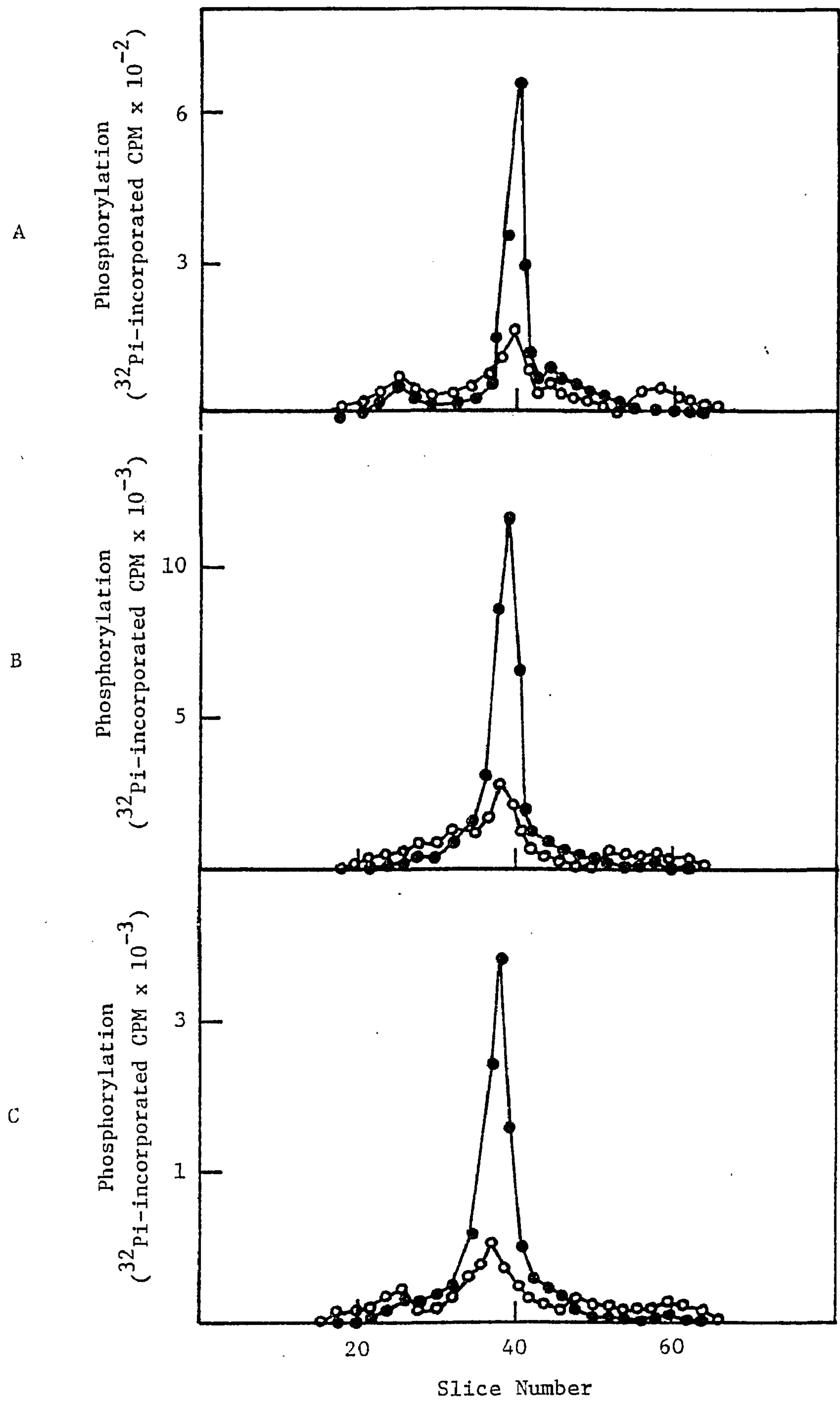


Fig. 37. Cyclic AMP-dependent dephosphorylation of phosphorylated plasma membrane, and protein kinases MPK II and CPK II. The cyclic AMP-dependent dephosphorylation of plasma membrane (part A) was carried out in an assay mixture containing [γ-^{32}P]-ATP (200 cpm/pmol), 200-300 μg of solubilized membrane, 50 mM Tris•HCl (pH 7.0), and 10 mM $MgSO_4$ in a total volume of 50 μl. After incubation for 5 minutes, 50 μl of 50 mM Tris•HCl (pH 7.0) was added to the control preparation, and 50 μl of a mixture containing 1 μM cyclic AMP and 1 mM theophylline was added to the experimental preparation. Incubation was continued for an additional 10 min at 30°C. The samples were subjected to SDS polyacrylamide gel electrophoresis by the method of Davis and Ornstein (206). The gel was then sliced into 1 mm sections, placed in toluene scintillation fluid and counted.

The assays for cyclic AMP-dependent dephosphorylation of protein kinase MPK II (part B) and CPK II (part C) were carried out by incubation of a mixture containing [γ-^{32}P]-ATP (100 cpm/pmol), 50 mM Tris•HCl (pH 7.0), 10 mM $MgSO_4$, and 50 μg of purified enzyme. After incubation for 5 min, 50 μl of a mixture containing 50 mM Tris (pH 7.0) and 50 μg purified protein phosphatase Pa or Ma was added to the control preparation, and 50 mM Tris•HCl (pH 7.0), 50 μg purified protein phosphatase Pa or Ma, and 1 μM cyclic AMP was added to the experimental preparation. Incubation was continued for an additional 10 min. The samples were subjected to SDS polyacrylamide gel electrophoresis as described above. The gel was then sliced into 1 mm sections, placed in toluene scintillation fluid, and counted.

In each part, the control reaction is indicated by ◦, and the cyclic AMP-dependent dephosphorylation, by ◦.

Fig. 38

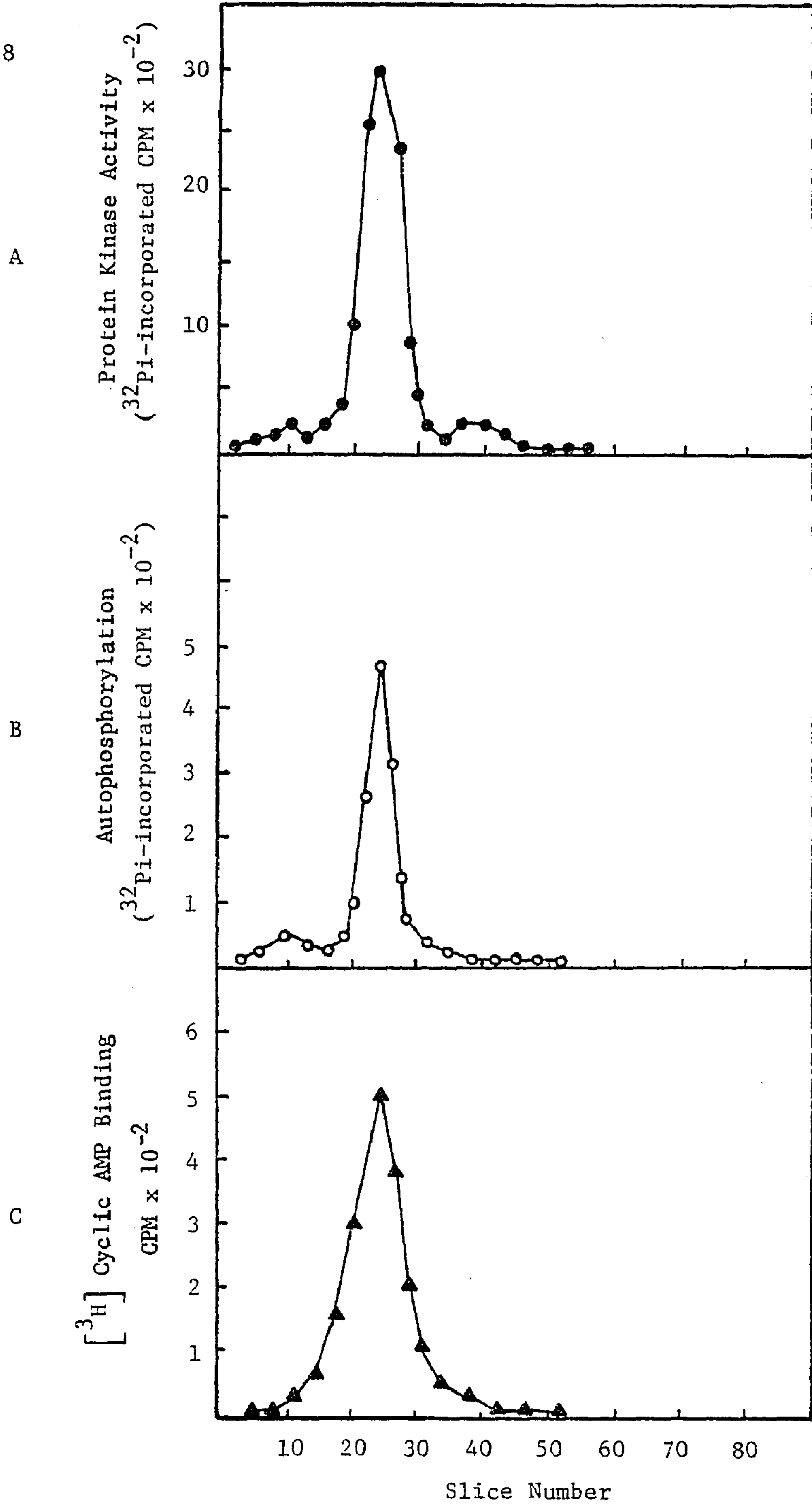


Fig. 38. <u>Polyacrylamide gel electrophoresis of membrane protein kinase MPK II</u>. The purified protein kinase MPK II was subjected to disc electrophoresis on 7% polyacrylamide gel by method of Davis and Ornstein (206). After the electrophoresis, the gel was sliced into 1 mm sections and assayed for protein kinase activity and [^{3}H]-cyclic AMP binding. The sliced sections were placed into the test tubes that had standard protein kinase assay mixture with a volume of 100 µl containing 50 mM TES (pH 7.0), 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]-ATP (100 cpm/pmol), 1 µM cyclic AMP and 100 µg histone. The standard [^{3}H]-cyclic AMP assay mixture contained 20 mM Tris•HCl (pH 7.0), 4 mM magnesium acetate, 20 µg/ml of BSA, and 40 nM [^{3}H] cyclic AMP (5 x 10^4 dpm/pmol). Both assays were carried out by the standard method described in Experimental Procedure section, incubated 20 min at 30°C. The autophosphorylation was carried out in an assay mixure of 100 µl containing 50 mM Tris•HCl (pH 7.0), 0.5 mM [γ-^{32}P] ATP (200 cpm/pmol), 50 µg of purified membrane protein kinase MPK II, 10 mM $MgSO_4$ and incubating 10 min at 30°C. The samples were subjected to SDS polyacrylamide gel electrophoresis by the method of Davis and Ornstein (206) and activity was detected by putting the sliced sections in toluene scintillation fluid and counting. In part A the protein kinase activity is indicated by •, in part B autophosphorylation by ∘, and in part C the [^{3}H] cyclic AMP binding by ▲.

Fig. 39

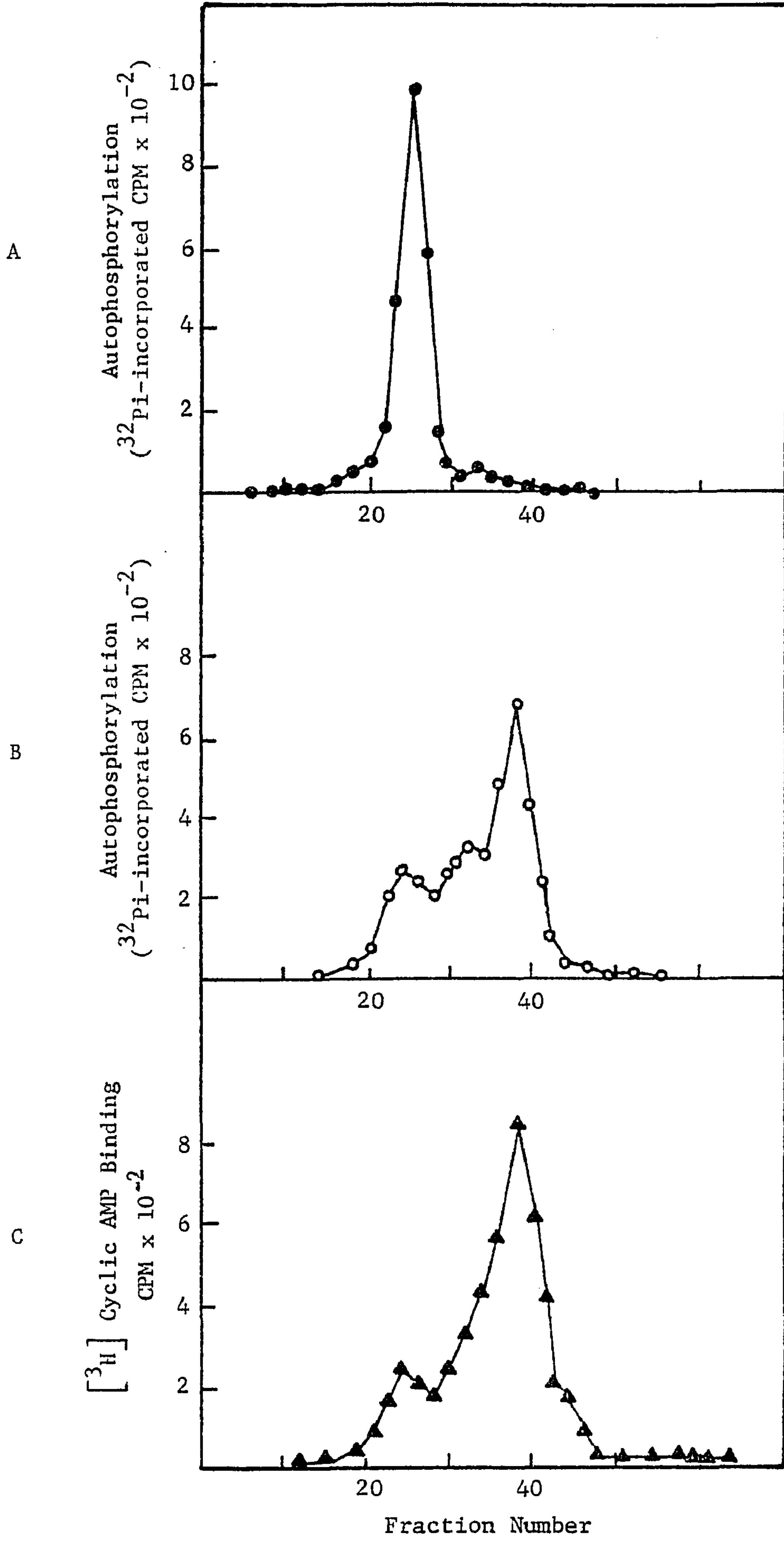


Fig. 39. Polyacrylamide gel electrophoresis of the holoenzyme and regulatory subunit of membrane protein kinase MPK II. The autophosphorylation of holoenzyme of protein kinase MPK II was carried out in a standard assay mixture of 100 μl containing 50 mM Tris·HCl (pH 7.0), [γ-^{32}P]-ATP (200 cpm/pmol) 50 μg of purified protein kinase MPK II, 10 mM $MgSO_4$, either in the presence or absence of 10 μM cyclic AMP. Incubation was for 10 min at 30°C. The samples were subjected to a disc electrophoresis on 7% polyacrylamide gel by the method of Davis and Ornstein. The gel was sliced into 1 mm sections, placed in toluene scintillation fluid and counted. The regulatory subunit (200 μg) isolated from CM-Sephadex C-50 described above was subjected to disc electrophoresis on 7% polyacrylamide by the method of Davis and Ornstein (206). After electrophoresis, the gel was sliced into 1 mm sections, placed into tubes containing the standard assay mixture for [^{3}H] cyclic AMP binding and incubated for 20 min at 30°C. The activity was detected by putting sliced sections in toluene scintillation fluid and counting. Part A indicates the autophosphorylation of holoenzyme MPK II, part B indicates the autophosphorylation of holoenzyme of MPK II in the presence of 10 μM cyclic AMP. Part C indicates the binding of cyclic AMP by the regulatory subunit MPK II.

Fig. 40(A)

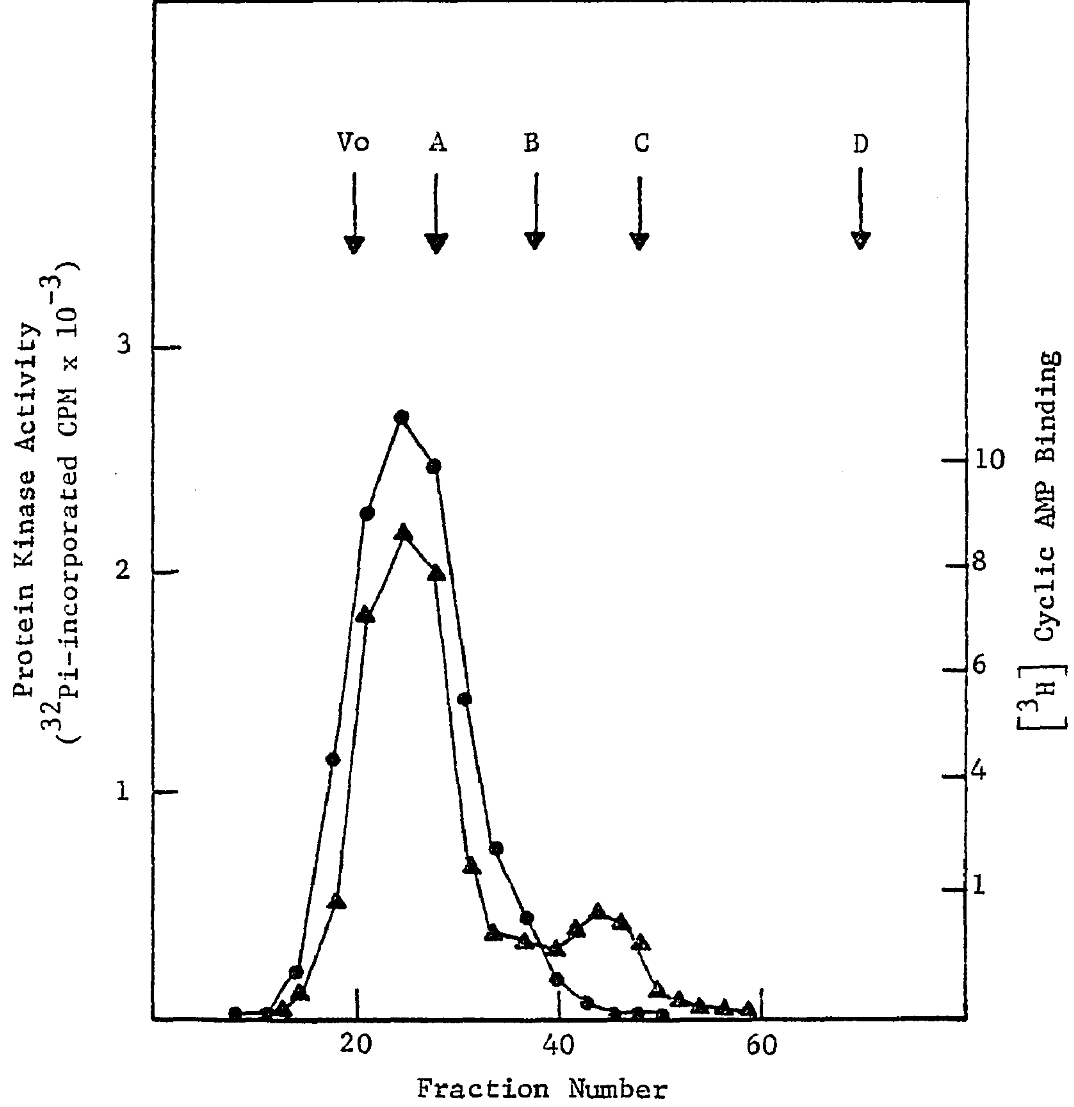


Fig. 40(B)

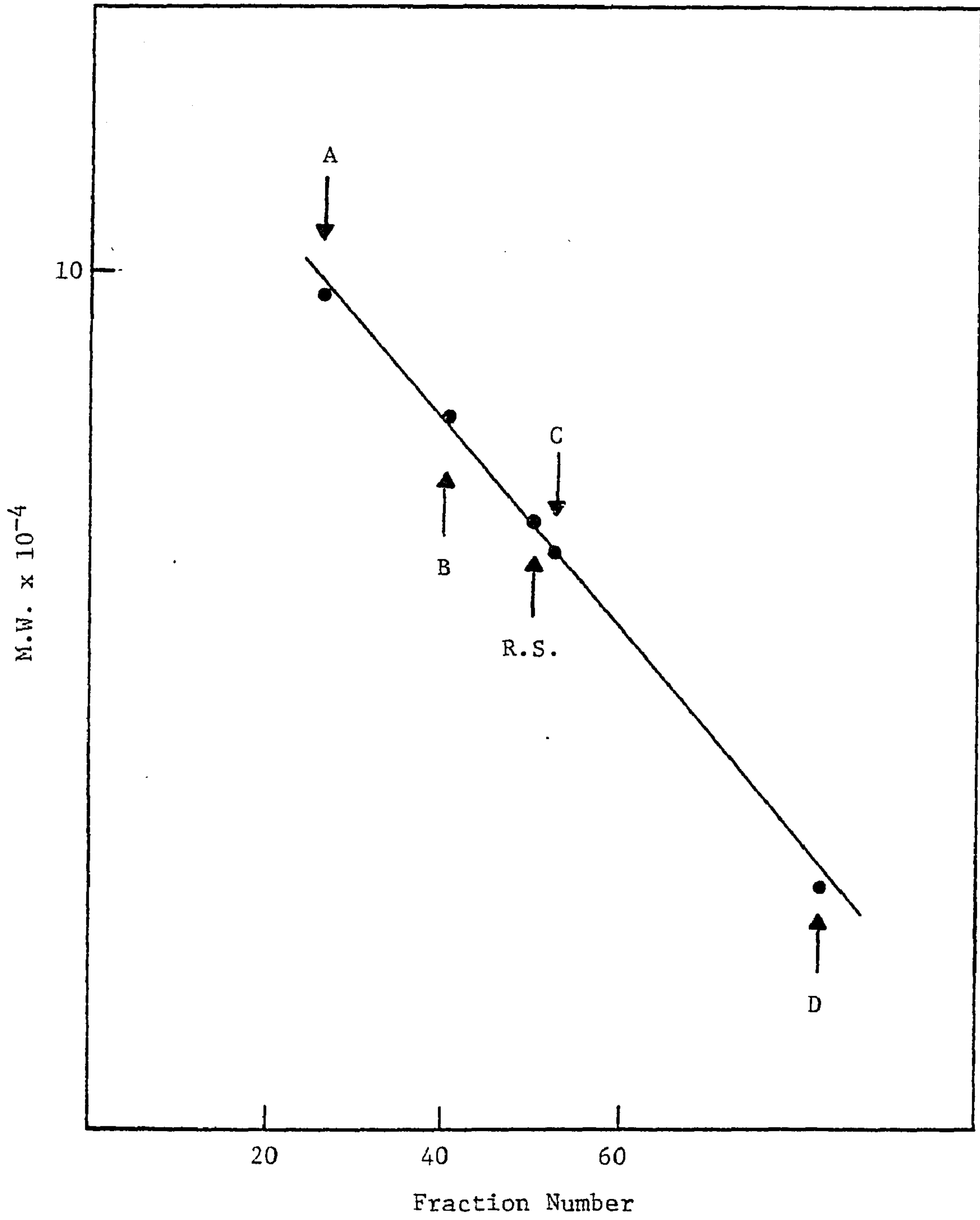


Fig. 40. Determination of the molecular weight of the regulatory subunit of membrane-bound protein kinase MPK II by Sephadex G-100 gel filtration. The regulatory subunit of membrane protein kinase MPK II obtained from CM-Sephadex C-50 column chromatography was concentrated by ultrafiltration through an Amicon PM-10 membrane and dialyzed against buffer A-T for 4 hr. The regulatory subunit (3 ml, 1.2 mg/ml) combined with the purified protein kinase MPK II (4 ml, 2.8 mg/ml) was filtered through a G-100 Sephadex column (1.6 x 64 cm) pre-equilibrated with buffer A-T. The protein kinase activity (•) and [^{3}H]cyclic AMP binding activity (▲) were determined by the standard method described above. The following proteins were used as internal standards for the determination of the molecular weight of the regulatory subunit: A, phosphorylase a (Mr = 94,000); B, bovine serum albumin (Mr = 67,000); C, ovalbumin (Mr = 43,000); D, myoglobin (Mr = 18,000). V_0 indicates the void volume.

Fig. 41

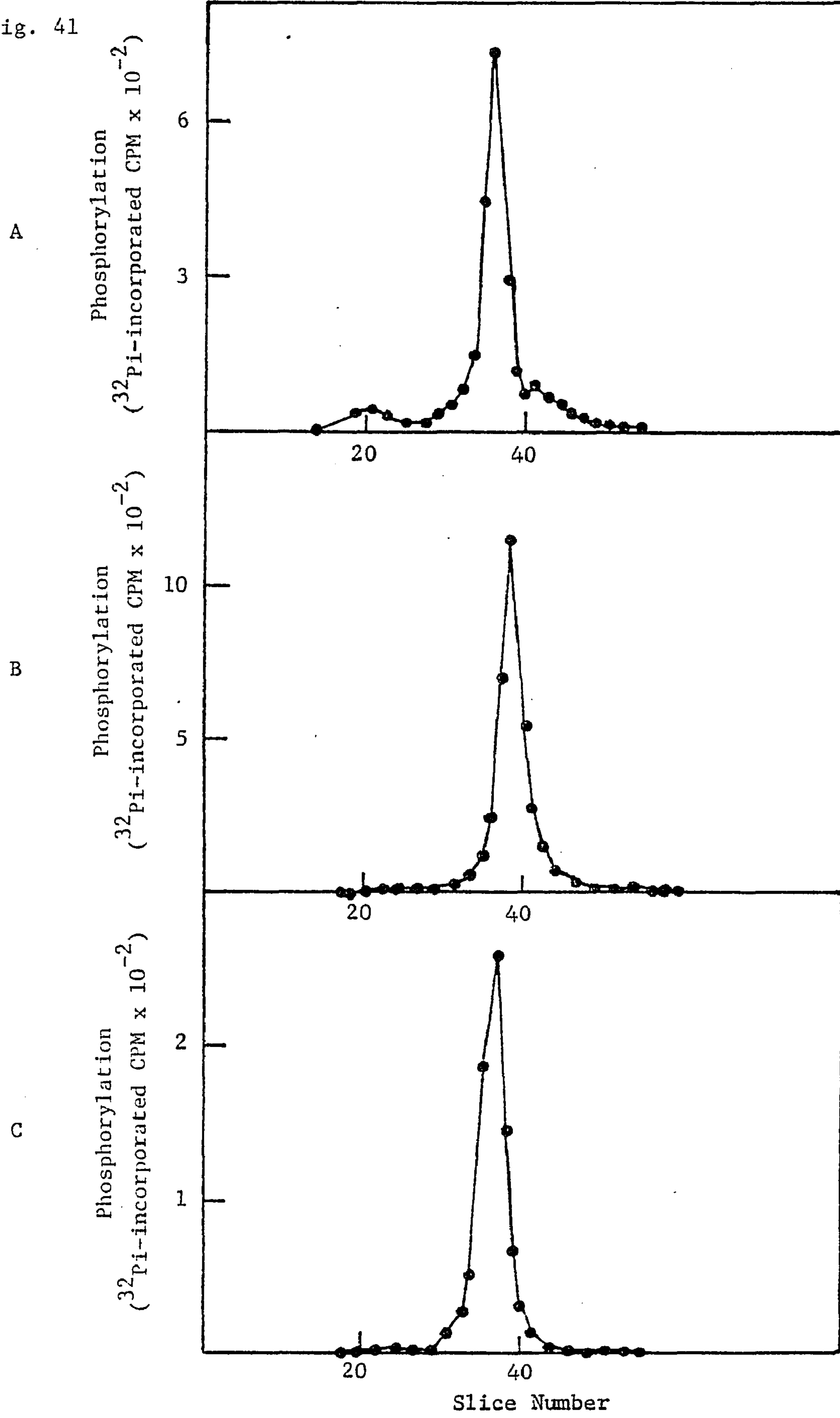


Fig. 41. Autophosphorylation of papillary plasma membrane and protein kinases MPK II and CPK II. The assay for autophosphorylation was carried out in a reaction mixture of 100 μl containing 50 mM Tris•HCl (pH 7.0) for MPK II or 50 mM potassium phosphate (pH 7.0) for CPK II, 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (100 cpm/pmol), 10% glycerol and 50 μg of enzyme. Incubation was performed for 10 min. at 30°C. The reaction was stopped by adding 1 ml of ice cold 40% trichloroacetic acid. The assay tubes were centrifuged in a table centrifuge for 1 min. The supernatants were discarded, the same volume of water added and the tubes recentrifuged. The pellets were suspended in 50 μl of SDS solubilizing solution, which was comprised of 2% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol, and 20 mM sodium phosphate (pH 7.0). After the sample was solubilized, 80 μl of solution B, which was comprised of 0.1 mM EDTA, 1% 2-mercaptoethanol, 20 mM sodium phosphate, 40% sucrose and 0.005% bromide blue, was added to the sample. Then 50 μl of the final solution was subjected to SDS gel electrophoresis. The electrophoresis was performed according to the procedure of Laemmli (207), employing a 10% polyacrylamide gel. The gel was sliced into 1 mm sections. The sliced sections were placed in toluene scintillation fluid and counted. Part A shows the phosphorylation of papillary plasma membrane, part B, that of membrane protein kinase MPK II, and part C, that of cytosol protein kinase CPK II.

Fig. 42. Autoradiography of cyclic AMP-dependent dephosphorylation of papillary plasma membrane

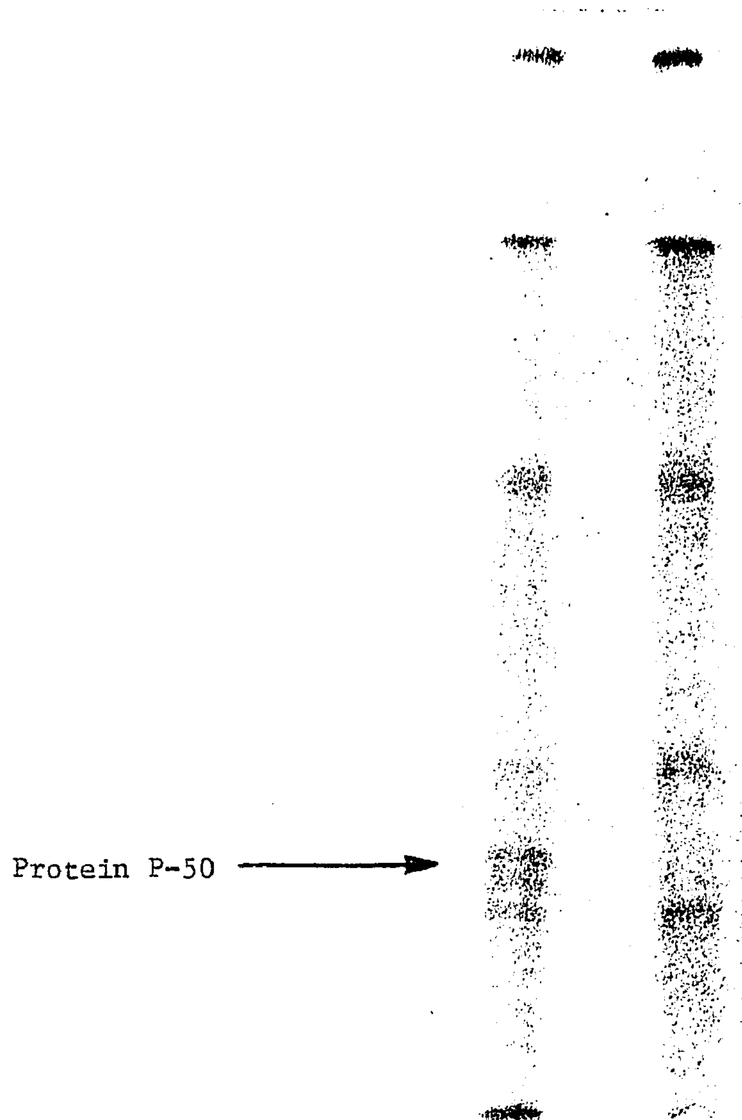


Fig. 42. Autoradiography of cyclic AMP-dependent dephosphorylation of papillary plasma membrane. The assay for autoradiography was carried out in a reaction mixture of 100 μl containing 50 mM 2-(N-Morpholino)Ethane-Sulfonic Acid (MES), 5 mM $ZnCl_2$, 10 mM $MgCl_2$, and 50 μM ATP (specific activity 500 cpm/pmol). Incubation was performed for 3 min at 30°, then 30 μl of a mixture which was comprised of a final concentration of 15 mM EDTA, 0.7 mM cold ATP, no cyclic AMP or 15 μM cyclic AMP was added, and incubation was continued for 10 minutes. The reaction was stopped with 1 ml of ice cold 10% TCA and 0.1 mM KPi. The assay tubes were centrifuged in a table centrifuge for 2 min. The supernatant was discarded, the same volume of water added, and tubes recentrifuged. The pellets were suspended in 50 μl of SDS solubilizing solution, which was comprised of 2% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol, and 20 mM sodium phosphate. After the sample was solubilized, 80 μl of solution B, which was comprised of 0.1 mM EDTA, 1% 2-mercaptoethanol, 20 mM sodium phosphate, 40% sucrose, and 0.005% bromide blue, was added to the sample. Then 100 μl of final solution was subjected to SDS-polyacrylamide gel electrophoresis.

The slab gel electrophoresis was carried out for 15 hr at 20-30 milliamps on a vertical plate gel of 7% polyacrylamide in the presence of 1% SDS. The gel was run by the method described by Fairbanks et al. (208). The gel dimensions were 11.5 cm x 12.8 cm x 1 mm, and the apparatus used was that described by Reid and Bieleski (209). The gel was stained for protein with 0.025% Coomassie Blue in 10% isopropyl alcohol, 10% acetic acid and destained with several changes

Fig. 42 (cont'd)

of 10% acetic acid. The stained gel was dried on Whatman No. 50 filter paper under vacuum and heat, and placed in close contact with Kodak Royal X-Omat film. The film was exposed for 10 days.

IV. DISCUSSION

This dissertation is concerned with the assessment and evaluation of the role of intracellular cyclic AMP in the action of ADH. In the following discussion, the results of the studies described above are considered in relation to the current knowledge of the action of ADH, and a hypothesis on the intracellular mechanism of action of ADH is presented.

1. Criteria for the Involvement of Phosphorylated Protein in the Cyclic AMP-Mediated Action of ADH

It has been generally accepted that the action of ADH is mediated by cyclic AMP, which serves as an intracellular second messenger (87, 213). However, the means by which cyclic AMP alters the permeabilities to water, sodium and urea is not yet completely understood.

In all mammalian tissues studied so far, the activation of protein kinase appears to be a major mechanism by which cyclic AMP carries out its function as a second messenger in the transmission of a hormonal signal (108). This observation led to the hypothesis that many of the actions of cyclic AMP in higher organisms are mediated by cyclic AMP-dependent protein kinases (105). It has also been observed that a number of hormones can raise the level of cyclic AMP in different tissues (103,213) but no high-affinity binding protein for cyclic AMP other than (a) the regulatory subunit of protein kinase and (b) phosphodiesterase has yet been identified in a eukaryotic cell (104, 108,109). Therefore, in order to formulate the mechanism of the intracellular action of ADH, one should take into consideration the following

proposition for the action of peptide hormones in general: cyclic AMP generated in response to peptide hormones exerts many of its effects in eukaryotic cells by activation of cyclic AMP-dependent protein kinase(s), thereby increasing the phosphorylation of proteins involved in the specific physiological responses to these hormones (40,105,109). Krebs has suggested five criteria that must be satisfied before an effect mediated by cyclic AMP can be said to occur via phosphorylation of a protein (106,108).

a. The cell type involved contains a cyclic AMP-dependent protein kinase.

b. A protein substrate exists which bears a functional relationship to the process mediated by cyclic AMP.

c. Phosphorylation of the protein substrate alters its function _in vitro_.

d. The protein substrate is modified _in vivo_ in response to cyclic AMP.

e. A phosphoprotein phosphatase exists to reverse the process.

In the following discussion, I will try to associate the results of my studies with the above five criteria to establish a hypothesis for the intracellular mechanism of the action of ADH.

The Canine Renal Medulla Protein Kinase System

In our studies (described above), the characteristics of the protein kinase enzymes system from renal medulla are summarized in the following table:

	Cytosol		Membrane	
	Peak I	Peak II	Peak I	Peak II
Cyclic AMP dependency	cAMP dependent	cAMP dependent	cAMP dependent	cAMP dependent
Concentration of salt used for elution from DE-52	<0.1 M KCl	<0.1 M KCl	<0.1 M KCl	<0.1 M KCl
Type	I (Rabbit skeletal muscle)	II (Bovine heart)	Membrane-bound	Membrane-bound
M.W.	180,000	170,000	200,000	105,000
% of total activity	20% of total cytosol P.K. activity	80% of total cytosol P.K. activity	20% of total membrane P.K. activity	80% of total membrane P.K. activity
Autophosphorylation	No	Yes	No	Yes
M.W. of regulatory subunit	50,000	55,000	-	50,000
Binding to [^{3}H]-cAMP	Yes	Yes	Yes	Yes
Characteristics	Soluble	Soluble	Membrane-bound	Membrane-bound

2. Cytosolic Protein Kinase

Our studies showed that the cells of the renal medulla have different protein kinase isoenzymes both in the cytosol and associated with membrane components. The cytosolic fraction has 65% of the total protein kinase activity. Elution by DEAE-cellulose ion-exchange chromatography showed two peaks of protein kinase activity in the cytosolic fraction. The minor peak represents 15% of total activity: the properties of this enzyme are similar to those of the type I protein kinase (CPK I) isolated from rabbit skeletal muscle by Krebs and his associates (123). The major peak represents 85% of total activity: the properties of this enzyme are similar to those of the type II protein kinase (CPK II) isolated from bovine cardiac muscle by Rosen and her coworkers (124, 131). The distinguishing characteristic of type II protein kinases is autophosphorylation (136,138), i.e., these kinases are able to phosphorylate their respective cyclic AMP-binding subunits. The studies of CPK II from bovine cardiac muscle indicated that the autophosphorylation of this enzyme has the following characteristics (135,137): (a) Phosphorylation changes the dissociation constant for cyclic AMP from 2.8 μM to 0.4 μM, i.e., it enhances the binding of cyclic AMP to the enzyme (136). (b) The phosphorylation of protein kinase enhances the net dissociation of inactive holoenzyme by cyclic AMP into its regulatory and active catalytic subunits. (c) The autophosphorylation also results in retardation of the rate of reassociation of its isolated subunits in the absence of cyclic AMP. From the results above, we know that approximately 85% of the renal cytosolic protein kinase activity is of type II. It is important to understand any functional

significance of this distribution for the action of ADH. Various studies from different tissues have demonstrated that the relative proportions of the two types of protein kinase in a given tissue can vary from species to species; this indicates that tissue-specific function cannot be deduced from the relative proportions of these types (108,109,133,134)

The kidney has a multiple hormonal receptor-adenylate cyclase system that responds to vasopressin, parathyroid hormone (PTH), calcitonin and catecholamines (214-219). Each hormone has its own receptor (coupled to adenylate cyclase) distributed in different sections of the renal tubule and regulating different renal functions. Morel and his colleagues have studied the hormone-sensitive adenylate cyclase activities in different segments of the rabbit nephron for each of the hormones mentioned above (218-223). The vasopressin-sensitive adenylate cyclase activities are located in the collecting tubules and the thick ascending limb of the loop of Henle (221). Parathyroid hormone caused marked stimulation of adenylate cyclase in cortex but only slight stimulation in medulla (220). The calcitonin-sensitive adenylate cyclase was found to be localized in the thick ascending limb of the loop of Henle (223). Thus, it is of interest that the segment which is mainly sensitive to calcitonin is insensitive to PTH, and the segment which is sensitive to PTH is insensitive to calcitonin. The isoproterenol-sensitive adenylate cyclase is distributed in the second portion of the distal convoluted tubule as well as in the cortical collecting tubules, regions in which ADH-sensitive adenylate cyclase (218) is also distributed in many species.

This distribution probably accounts for the finding that β-adrenergic agents have an antidiuretic action on the mammalian kidney (218,224,225).

In canine renal tissue, we have demonstrated that the corresponding type I and type II cytosolic protein kinases not only have the same characteristics in the cortex and medulla but the relative proportions of these two types are similar in these two regions. Also the finding that cytosolic protein kinase activity has the same distribution throughout the renal tubule suggests that the presence of these soluble protein kinases is not a specific feature of the ADH target cell.

3. Membrane-Bound Protein Kinases

In various studies, it has been shown that the action of ADH occurs at the apical membrane of the target cell (167) and that this hormone acts by affecting a water permeability barrier located in this membrane. The characteristics of membrane-bound protein kinase (discussed below) suggest that one or more of these enzymes may play a critical role in the ADH-induced change in membrane permeability.

The major membrane-bound cyclic AMP-dependent protein kinase, MPK II, which we isolated from canine papillary plasma membrane, has the following characteristics: (a) The enzyme represents less than 5% of the total protein kinase activity in the ADH target cell. (b) The enzyme is tightly bound to membrane and is unstable after solubilization; the cyclic AMP-dependent activity of this enzyme is greatly enhanced by solubilization. (c) The enzyme can phosphorylate exogeneous substrates such as histone, protamine, casein and intrinsic membrane protein substrate as well. (d) The molecular weight of the enzyme is

approximately 100,000; the enzyme is comprised of one catalytic subunit and one regulatory subunit; the molecular weight of the latter subunit has been found to be approximately 50,000. (e) The enzyme autophosphorylates its cyclic AMP-binding subunit.

The characteristics of the major component of membrane-bound protein kinase (MPK II) from the renal medullary tissue indicate that it is different from the major soluble cyclic AMP dependent protein kinase (CPK II) from the same cells in several respects. The molecular weight of CPK II is 180,000. The same type of kinase isolated from bovine cardiac muscle is comprised of a regulatory cyclic AMP-binding dimer having a total molecular weight of 100,000 (124, 131) and two catalytic subunits, each with a molecular weight of 38,000 (124,131). On the other hand, MPK II from canine renal medulla has a molecular weight of 100,000, approximately half that of CPK II, and is similar to protein kinase having one catalytic and one regulatory subunit isolated from neural membrane (139). It is also noteworthy that, according to Greengard and his coworkers (139), both the catalytic and the regulatory subunits of the membrane-bound protein kinase isolated from neural membrane differ from those of CPK II isolated from brain.

Both cytosolic type II protein kinase (CPK II) and the major membrane-bound protein kinase (MPK II) that we obtained from canine renal medulla exhibit autophosphorylation. MPK II is very tightly associated with membrane; it cannot be removed without detergent. Thus, the mobility of this enzyme is probably restricted in vivo. In comparison to membrane-bound enzyme, cytosolic protein kinase is mobile intracellularly. The time course of activation of the

cytosolic protein kinase is short compared to that of the membrane-bound enzyme, as shown in the studies presented above. Because the cytosolic enzyme represents approximately 65% of the total activity, one might think that it might play a major role in the mediation of the action of ADH. However, the morphological distribution of the enzyme must also be taken into consideration. Schwartz _et al._ (99) purified the luminal and contraluminal plasma membranes from bovine renal papilla by using free-flow electrophoresis. The contraluminal, but not the luminal, membrane was found to contain ADH-sensitive adenylate cyclase. The luminal membrane was found to contain a cyclic AMP-sensitive, endogenous phosphorylation system consisting of a membrane-bound protein kinase and its membrane-bound substrate(s); this intrinsic protein kinase was not present in the contraluminal membrane (99). These findings provide direct evidence in support of a model for the action of ADH on the kidney in which the initiating steps take place at the contraluminal pole of the hormone-sensitive target cell and the late or terminal steps occur at the luminal pole, where they involve an alteration in the level of membrane phosphorylation.

4. Cyclic AMP-Dependent Dephosphorylation and Membrane-Bound Protein Kinase Substrate

In the studies presented above, we also found the cyclic AMP-dependent dephosphorylation of a protein of molecular weight 50,000 associated with renal papillary plasma membrane. DeLorenzo _et al._ (147,148) demonstrated that in ADH-sensitive toad bladders prelabelled with $^{32}P_i$, then exposed to ADH, a microsomal protein of molecular

weight 50,000 was dephosphorylated. A similar result was obtained when the toad bladder was exposed to monobutyryl cyclic AMP. This effect was attributed to the enhancement by cyclic AMP of protein phosphatase activity in the particulate fraction (148). The dephosphorylation of this protein of molecular weight 50,000, referred to as protein D, preceded the increase in sodium transport measured simultaneously in a paired control hemibladder (147) and paralleled the water permeability response to vasopressin (150). Various other agents which affect sodium transport and water permeability in toad bladder have also been shown to increase the dephosphorylation of protein D (147-149). Therefore, it was proposed that protein D may mediate the ADH-stimulated, cyclic AMP-mediated water permeability change (150). Greengard and his colleagues (149) subsequently reported that the cyclic AMP-dependent dephosphorylation of a protein similar or identical to protein D occurs in all the vertebrate tissues examined; this protein was then given the general name, protein P-50.

There are several possible roles of protein P-50 in regulating transport in the ADH target cell. Protein P-50 might be a plasma membrane-bound protein, the cyclic AMP-dependent dephosphorylation of which is directly involved in the effect of ADH on water and sodium transport. We believe this to be unlikely because the cyclic AMP-dependent dephosphorylation of protein P-50 can be detected in every tissue which has been studied (147) and many of these tissues are not involved in hormone-mediated transport phenomena. In other words, the cyclic AMP-dependent dephosphorylation of protein P-50 does not occur exclusively in ADH-sensitive tissues. Because only

the distal nephron, in the case of mammalian tissues, can respond to ADH, it is unlikely that a widespread non-specific phenomenon such as cyclic AMP-dependent dephosphorylation of protein P-50 would be responsible for the characteristic ADH-induced change in membrane permeability to water. Alternatively protein P-50 could be an integral part of the cyclic AMP-activated protein kinase system that is present in all mammalian tissues including those that are insensitive as well as those that are sensitive to ADH (149-151).

We found that cyclic AMP-dependent dephosphorylation of protein P-50 occurred in renal papillary plasma membrane (PPM), partially purified membrane-bound protein kinase (MPK II), and partially purified renal cytosolic protein kinase (CPK II). (It should be noted that the method used in our studies differs from that of Greengard and his coworkers in that they prelabelled with [$^{32}P_i$] intact tissue to demonstrate the dephosphorylation of protein P-50 (147) whereas we used [γ-^{32}P]ATP to label the prepared substrates in isolated systems).

In the examination of cyclic AMP-dependent dephosphorylation in PPM, no protein phosphatase was added because the PPM has membrane-bound protein phosphatase activity. On the other hand, in the examination of the dephosphorylation of membrane-bound protein kinase (MPK II) and renal cytosolic protein kinase (CPK II), protein phosphatase had to be added in order to initiate dephosphorylation.

A notable aspect of the cyclic AMP-dependent dephosphorylation of MPK II and CPK is the fact that although cyclic AMP does bring about dephosphorylation of protein P-50, the activity of the added

protein phosphatase was not altered by cyclic AMP when phosphohistone, phosphocasein and phosphomembrane were used as substrates.

In view of the widespread occurrence of protein P-50 and the effect on it of cyclic AMP, we sought to determine the identity of this protein and the significance of its state of phosphorylation. We were led to conclude that protein P-50 of toad bladder and other tissues is the cyclic AMP-binding regulatory subunit of protein kinase.

In order to determine definitively that protein P-50 is the regulatory subunit of cyclic AMP-dependent protein kinase, the following requirements must be satisfied: (a) Protein P-50 must be of the same molecular weight as the regulatory subunit. (b) Protein P-50 must bind to cyclic AMP, and the characteristics of this binding must be similar to those possessed by the regulatory subunit. (c) Protein P-50 should suppress the activity of the catalytic subunit of protein kinase. (d) Protein P-50 should have the same immunochemical properties as the regulatory subunit of protein kinase.

There are four findings that suggest that protein P-50 could be this subunit: (a) Protein P-50 binds to the photo-affinity cyclic AMP analogue, 8-azido-cyclic AMP, which also binds to the regulatory subunit of protein kinase (151). (b) The molecular weight of protein P-50 (49,000-50,000) is the same as that of the regulatory subunit of bovine cardiac protein kinase. (c) Protein P-50 and cyclic AMP-dependent protein kinase have been found in every mammalian tissue studied. (d) Cyclic AMP-dependent dephosphorylation of protein P-50 also has been found to be associated with CPK II and MPK II, both of which undergo autophosphorylation.

The binding of cyclic AMP by P-50 strongly suggests that this protein is the regulatory subunit of protein kinase because no high affinity cyclic AMP-binding protein other than the regulatory subunit of cyclic AMP-dependent protein kinase and phosphodiesterase has been identified in eukaryotic organisms (108, 109). Phosphodiesterase hydrolyses the cyclic AMP to 5'-AMP; it does not covalently bind 8-azido-cyclic AMP, the photo-affinity analogue (151), as does the regulatory subunit. Other cyclic-AMP binding proteins isolated from liver and erythrocytes have high molecular weights, viz., 180,000 and 240,000, respectively (226,227), and bind the cyclic nucleotide with low affinity.

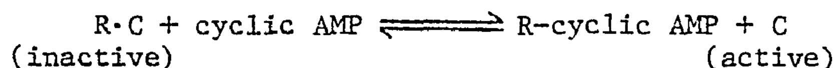
To fulfill the third and and fourth requirements for definitive identification of P-50 as R, one would have to purify P-50. Although this purification has not yet been accomplished, we have obtained the following evidence suggesting that this protein is indeed the regulatory subunit of protein kinase: Sephadex G-200 gel filtration of solubilized renal plasma membrane protein shows only three regions of molecular weight in which proteins exhibits binding to cyclic AMP, namely 240,000, 200,000 and 100,000. The protein(s) in the regions of molecular weight 200,000 and 100,000 correspond to protein kinase MPK I and MPK II, respectively. The proteins in the region of molecular weight 240,000 bind cyclic AMP but show no cyclic AMP-dependent protein kinase activity or protein phosphatase activity. No other region was found to bind cyclic AMP. In the region of molecular weight 50,000 (the region that should contain the regulatory subunit R if it were present), no cyclic AMP-binding activity was found --- indicating that protein P-50 arises only by dissociation from a larger molecule.

From this Sephadex G-200 column, we separated the solubilized renal plasma membrane protein into three fractions: fraction I containing proteins with molecular wieghts of 30,000-70,000, fraction II contained protein with molecular weights of 70,000-140,000, and fraction III contained proteins with molecular weights larger than 140,000. Fraction I, which includes the region of molecular weight 50,000, did not show either autophosphorylation or binding to [^{3}H]cyclic AMP. Only fraction II, which contained membrane-bound protein kinase MPK II, exhibited autophosphorylation and binding to [^{3}H]cyclic AMP. Fraction III exhibit binding to [^{3}H]cyclic AMP but not autophosphorylation. In the presence of cyclic AMP, however, it did phosphorylate histone, therefore fraction II containes the type II protein kinase. Adding [γ-^{32}P]ATP to each fraction and then subjecting the fraction to SDS-polyacrylamide gel electrophoresis showed that only fraction II had a phosphorylated peak of molecular weight 50,000. This phosphorylated peak was dephosphorylated in the presence of cyclic AMP and protein phosphatase. When purified protein kinase MPK II was purified and subjected to SDS-polyacrylamide gel electrophoresis it was found that cyclic AMP induced the dephosphorylation of a protein of a molecular weight 50,000; this protein also bound cyclic AMP and therefore was tentatively identified as the regulatory subunit of protein kinase MPK II. Because fraction I showed neither autophosphorylation nor cyclic AMP-dependent dephosphorylation, there was clearly no R in this protein. However, because a protein of molecular weight 50,000 (P-50) from fraction II did bind [^{3}H]-cyclic AMP and did manifest autophosphorylation, it appear that this protein must have dissociated from a large molecule, i.e. on electrophoresis of the protein kinase holo-

enzyme, a smaller phosphorylated protein (M_r = 50,000) appeared on the gel.

5. Mechanism of Cyclic AMP-Dependent Dephosphorylation of Protein P-50.

All the cyclic AMP-dependent protein kinases examined have the general quarternary structure $R_n \cdot C_n$ (108), where R is the regulatory subunit (a cyclic AMP-binding protein), C is the catalytic subunit, and n = 1 or 2. The holoenzyme is inactive; when cyclic AMP binds to the regulatory subunit, it promotes dissociation of holoenzyme; the free catalytic subunit is active. When n = 1, the reaction scheme may be written as follows:



$$\underset{\text{(inactive)}}{R\cdot C} + \text{cyclic AMP} \rightleftharpoons \text{R-cyclic AMP} + \underset{\text{(active)}}{C}$$

In order to define the mechanism of cyclic AMP-dependent dephosphorylation, the following questions must be answered: (a) How can the apparent contradiction between cyclic AMP-stimulated phosphorylation and cyclic AMP-stimulated dephosphorylation be explained? (b) What is the function of cyclic AMP-dependent dephosphorylation? (c) Is cyclic AMP involved in the regulation at the enzyme level or at the substrate level?

We were able to conclude that cyclic AMP-dependent dephosphorylation does not take place at the enzyme level, since none of the purified phosphoprotein phosphatases bound cyclic AMP or were activated by cyclic AMP directly (138,144,-28-232). The regulatory subunit of protein phosphatase from liver also did not bind cyclic AMP (144,229,232). Therefore, it would appear that no direct interaction exists between cyclic AMP and phosphatase either in the case of renal cytosolic or renal membrane-bound phosphatases or for that matter in the studies of liver phosphatases noted above.

If cyclic AMP-dependent dephosphoryaltion is regulated at the substrate level, one must consider how the binding of cyclic AMP to its target molecule activates the dephosphorylation. The bound cyclic AMP could be an allosteric effector, or it could serve to release the protein P-50 from its attachment to another protein in the manner discussed above for the action of cyclic AMP on the protein kinase holoenzyme. A model for the allosteric effect of cyclic AMP on a high-affinity cyclic AMP-binding protein is provided by phosphodiesterase (108), an enzyme which is activated in the presence of cyclic AMP and then hydrolyzes cyclic AMP to 5' AMP. However this is not analogous to the interaction of cyclic AMP and protein P-50, for the latter does not utilize cyclic AMP as substrate. It is well established that the binding of cyclic AMP to a protein kinase holoenzyme, R·C, results in the dissociation of the regulatory subunit, R, from R·C. Therefore, because of the evidence that protein P-50 is R, we are suggesting that cyclic AMP binds to P-50 (R) releasing it from R·C. In turn, the release of P-50 from holoenzyme, in the presence of protein phosphatase(s) greatly enhances the susceptibility to phosphoprotein phosphatase of the phospho-serine and/or phospho-threonine residues of P-50. It appears, therefore, that the phosphorylated residues of protein P-50 are less accessible to protein phosphatases when P-50 is associated with the catalytic subunit in the holoenzyme. That the accessibility of P-50 to protein phosphatase is increased when the P-50-cyclic AMP complex is released from the holoenzyme has been noted in a bovine cardiac muscle system (138). Thus, it appears that the action of cyclic AMP to induce the dephosphorylation of P-50 is regulated at the substrate level.

Rangel-Aldao and Rosen (233) recently studied the mechanism of autophosphorylation of cyclic AMP-dependent protein kinase from bovine cardiac muscle and concluded that the undissociated holoenzyme does not catalyze the phosphorylation of exogeneous substrate but can undergo autophosphorylation by an intramolecular reaction. The following results of their study are noteworthy: (a) addition of either cyclic AMP-binding protein or heat-stable protein kinase inhibitor does not inhibit autophosphorylation (the latter inhibits the phosphorylation of exogeneous substrate in the presence or absence of cyclic AMP); (b) addition of catalytic subunit to an excess of cyclic AMP-binding protein results in phosphorylation equivalent to the amount of holoenzyme generated; (c) the rate of autophosphorylation is not affected by dilution of the holoenzyme (233).

The autophosphorylation of bovine cardiac muscle protein kinase may be stimulated by cyclic AMP, but this stimulation was observed only at low concentration of ATP, viz., 50 μM (233). In any case, autophosphorylation of protein kinase holoenzyme is not dependent on cyclic AMP, as evidenced by the fact that it still occurs in the absence of this cyclic nucleotide.

We considered it important to determine whether dephosphorylation of P-50 occurs in the absence of cyclic AMP and to what degree the involved protein phosphatase has substrate specificity.

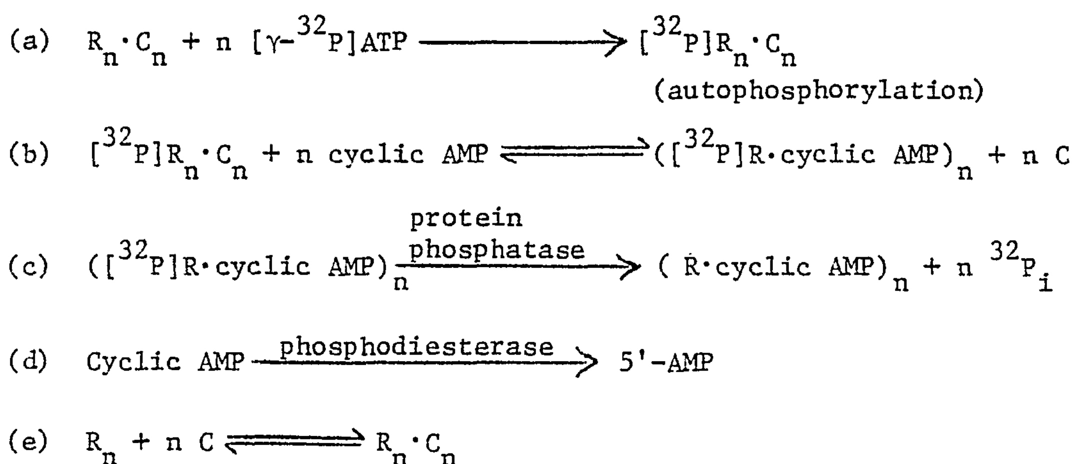
Chou and Rosen (138) recently purified a protein phosphatase (M_r = 30,000) from bovine cardiac muscle. The enzyme catalyzes dephosphorylation of cyclic AMP-binding protein of protein kinase. The action of this phosphatase on phosphorylated protein kinase, but not on other phosphoprotein substrates, was cyclic AMP-dependent. The

dephosphorylation of phosphorylated protein kinase was greatly enhanced upon increasing cyclic AMP from 0.1 μM to 10 μM. On the other hand, the dephosphorylation of phosphohistone was not affected by changing the concentration of cyclic AMP. This selective activation by cyclic AMP reflects the preference of the phosphatase for the free phosphorylated cyclic AMP-binding protein rather than the phosphorylated holoenzyme (138). Since autophosphorylation of protein kinase occurs by an intramolecular reaction and at a very low concentration of ATP, it is likely that under most physiological conditions, the bovine cardiac muscle holoenzyme exists as a phosphoprotein and it is not surprizing that a phosphatase acts on the dissociated phosphorylated regulatory subunit rather than on the phosphorylated protein kinase holoenzyme (138). This specificity for the free regulatory subunit was also exhibited by bacterial alkaline and potato acid phosphatase when phosphorylated cyclic AMP-binding protein and phosphorylated holoenzyme were subjected to these enzymes (138). All of these findings suggest that the catalytic site of C may be buried inside the holoenzyme, permitting phosphorylation of R to occur by an intramolecular reaction but preventing the holoenzyme from catalyzing the phosphorylation of exogeneous substrates (138).

Since modification of protein by phosphorylation and dephosphorylation are important processes, it may also be essential to determine whether each dephosphorylation reaction is catalyzed by a specific phosphatase or there is coordinate regulation of the dephosphorylation of a group of proteins analogous to the phosphorylation reaction catalyzed by protein kinase. The purified bovine cardiac muscle protein phosphatase exhibits broad substrate specificity both in the large molecular weight and ethanol-activated small molecular weight

form (230,234,235). This specificity is compatible with previous observations on phosphorylase phosphatase and glycogen synthetase phosphatase isolated from various sources (204,228,236-240). Recently, Killila _et al._ (228) provided evidence that glycogen synthetase and phosphorylase are coordinately regulated by a single protein phosphatase purified from rabbit liver.

From the above discussion, the following scheme may explain the mechanism of the cyclic AMP-dependent dephosphorylation of P-50



(a) $R_n \cdot C_n + n\ [\gamma\text{-}^{32}P]ATP \longrightarrow [^{32}P]R_n \cdot C_n$ (autophosphorylation)

(b) $[^{32}P]R_n \cdot C_n + n$ cyclic AMP $\rightleftharpoons ([^{32}P]R \cdot \text{cyclic AMP})_n + n\ C$

(c) $([^{32}P]R \cdot \text{cyclic AMP})_n \xrightarrow{\text{protein phosphatase}} (R \cdot \text{cyclic AMP})_n + n\ ^{32}P_i$

(d) Cyclic AMP $\xrightarrow{\text{phosphodiesterase}}$ 5'-AMP

(e) $R_n + n\ C \rightleftharpoons R_n \cdot C_n$

Step a. Autophosphorylation of holoenzyme ($R_n \cdot C_n$) to phosphorylated holoenzyme ($[^{32}P]R_n \cdot C_n$).

Step b. In the presence of cyclic AMP at physiological concentration (1 μM), the phosphorylated holoenzyme ($[^{32}P]R_n \cdot C_n$) dissociates into phosphorylated cyclic AMP-binding subunit (phosphorylated protein P-50) ($([^{32}P]R \cdot \text{cyclic AMP})_n$) and catalytic subunit (C).

Step c. The cyclic AMP-binding subunit ($([^{32}P]R \cdot \text{cyclic AMP})_n$) then is dephosphorylated by protein phosphatase to dephosphorylated cyclic AMP-binding subunit ($(R \cdot \text{cyclic AMP})_n$) and inorganic phosphate ($^{32}P_i$).

Step d. Cyclic AMP is hydrolyzed by phosphodiesterase to 5'-AMP. Thus, the concentration of cyclic AMP falls to a very low level, and free cyclic AMP-binding subunit (R_n) is produced when the bound cyclic AMP dissociates.

Step e. The dephosphorylated protein P-50 (R_n) and catalytic unit (C) reassociate to form the dephosphorylated holoenzyme ($R_n \cdot C_n$).

In our studies, the purified cytosolic and membrane-bound type II protein kinase from the canine renal medulla (CPK II and MPK II, respectively) both showed cyclic AMP-dependent dephosphorylation in the presence of protein phosphatase. Our results can be seen in the framework of the above reaction mechanism as follows:

Step a. CPK II and MPK II are autophosphorylated in the presence of [γ-^{32}P]ATP. In the absence of protein phosphatase cyclic AMP at physiological concentrations slightly increased the autophosphorylation.

Step a and b. Autophosphorylation resulted in ^{32}P-labelling of the regulatory subunit, which was dissociated from the holoenzyme upon binding cyclic AMP.

Step b. In the absence of cyclic AMP, protein phosphatase could not dephosphorylate the phosphorylated holoenzyme of protein kinase because step b could not proceed, thereby preventing step c from being realized (the phosphorylated holoenzyme is a poor substrate for phosphoprotein phosphatase).

Step b and c. In the presence of a physiological concentration of cyclic AMP (1 μM - 10 μM), the phosphorylated regularory subunit dissociated from the holoenzyme and was then dephosphorylat-

ed by protein phosphatase (i.e., the free phosphorylated subunit is a good substrate for protein phosphatase).

Thus we are suggesting that the protein P-50 that is (a) derived from the plasma membrane of ADH-sensitive renal medulla and (b) dephosphorylated in the presence of cyclic AMP is in all likelihood the regulatory subunit of membrane-bound protein kinase (MPK II).

6. Protein Phosphatases.

The following discussion concerns the protein phosphatase(s) of ADH-sensitive renal tissue with particular reference to the fifth criterion of Krebs.

The protein phosphatase enzyme system(s) can be regarded as a device for terminating the action of cyclic AMP by the catalytic removal of the phosphate group from the phosphorylated regulatory proteins. The exact nature of regulation of the activity of protein phosphatases is unknown. The current knowledge of protein phosphatases comes from studies of glycogen metabolism, e.g., phosphorylase phosphatase and glycogen synthetase phosphatase (235-241). Although some have suggested that cyclic AMP is directly involved in the regulation of these enzymes, this does not appear to be the case in most tissues that have been studied (204,230,236-242). For example, Huang and Glinsman (243,244) reported that a partially purified phosphorylase phosphatase from rabbit muscle can be inactivated by a phosphorylation reaction catalyzed by cyclic AMP-dependent protein kinase and that this inactivation is accompanied by a decrease in the molecular weight of the phosphorylase phosphatase from 70,000 to 52,000 and by the formation of phosphorylated inhibitory protein with molecular weight of 26,000. Dephosphorylation of this

inhibitor results in the loss of its inhibitory action toward phosphorylase phosphatase. It should be noted, however, that the inhibitor in its phosphorylated state does not affect the catalytic action of phosphorylase phosphatase on the dephosphorylation of phosphohistone or muscle glycogen synthetase D (244). In addition to the <u>phosphorylatable</u> protein inhibitor (M_r = 26,000), they also isolated a <u>non-phosphorylatable</u> protein inhibitor (M_r = 32,000) from skeletal muscle. The exact physiological role of these heat-stable inhibitors in the regulation of protein phosphatase is yet to be established.

In our studies of renal protein phosphatase, we did not find any direct action of cyclic AMP on this enzyme; the only involvement of cyclic AMP in the regulation of protein phosphatase activities is the cyclic AMP-stimulated dephosphorylation of protein P-50, the mechanism of which was explained above; this effect of cyclic AMP is an example of regulation at the substrate level.

Multiple forms of protein phosphatase have been demonstrated with different substrates in various tissues (235-245). It has been found that the multiple forms of protein phosphatase can be dissociated by treatment with either 2-mercaptoethanol (236,245) or ethanol (230,234) into a single catalytic subunit, thereby activating the enzyme. Li *et al*. (230,235) have proposed that the multiple forms of phosphorylase phosphatase consist of an enzyme-inhibitory protein complex which contains a single common catalytic subunit of molecular weight 32,000 and different inhibitory proteins that are removed by complexation with various specific metabolites.

In the early studies of muscle phosphorylase phosphatase, Graves _et al_. (246) found a specific protein phosphatase that acted only on phosphorylase _a_ or on a phosphopeptide derived from phosphorylase _a_. Originally, it was assumed that each phosphoprotein was dephosphorylated by a specific protein phosphatase. This assumption is important because if this is true, we probably would not be able to isolate the assumed cyclic AMP-stimulated phosphatase for protein P-50 until we first isolate protein P-50 itself. As was noted above, protein P-50 is either the regulatory subunit of protein kinase (as we believe to be the case) or a different protein which we were not able to separate from the regulatory subunit. Recently, however, the studies on the glycogen synthetase phosphatase from rabbit skeletal muscle (231), histone phosphatase from bovine cardiac muscle (138). and homogeneous phosphorylase phosphatase from rabbit liver (247) have demonstrated that a single protein phosphatase can catalyze the dephosphorylation of several phosphoproteins. The substrates include glycogen synthetase D, phosphorylated phosphorylase kinase, phosphorylase _a_, and phosphorylated protein kinase. By contrast, Kikuchi _et al_. (248) have recently reported that glycogen synthetase phosphatase can be clearly separated from phosphorylase phosphatase activity in rat liver. Furthermore, Antonie and Cohen (249) have reported the separation of two phosphorylase kinase phosphatase activities from rabbit skeletal muscle; one is specific for the dephosphorylation of the α-subunit of phosphorylase kinase.

In renal tissue, when using phosphorylated histone, casein and phosphorylase _a_ as substrates, we found two different protein

phosphatases in cytosol characterized by molecular weights of 105,000 and 31,000. Protein phosphatase Pb (M_r = 105,000), which is unstable _in vitro_, was easily converted to the catalytic subunit of protein phosphatase Pa (M_r = 31,000). Protein phosphatase Pa did not demonstrate substrate specificity, but it dephosphorylated phosphorylated membrane; it seems to be very similar to the catalytic subunit of phosphorylase phosphatase isolated from liver (247). An interesting aspect of the membrane-bound phosphatase is that the enzyme also has two isoenzyme, characterized by molecular weights of 100,000 and 35,000. Treatment of enzyme Mb (M_r = 100,000) with ethanol converts it into Ma (M_r = 35,000) with a concomitant increase in activity. The membrane-bound protein phosphatase is neither cyclic AMP-dependent nor very tightly bound to membrane; the affinity of this enzyme is different from that of membrane-bound protein kinase, which _is_ very tightly bound to membrane. The properties of membrane-bound phosphoprotein phosphatase, Ma, are very similar to those of cytosol protein phosphatase, Pa. It is quite possible that these two enzymes are one and the same; however we cannot prove this point until we purify the enzyme to homogeneity. Although we were able to isolate protein phosphatases from membrane and cytosolic fractions, we did not find any cyclic AMP dependence of these enzymes. However when we used either membrane-bound phosphatase or cytosolic phosphatase, we were able to demonstrate cyclic AMP-dependent dephosphorylation of the phosphorylated regulatory subunit (P-50) of protein kinase.

If phosphorylation of the apical membrane of the mammalian renal collecting duct has a role in ADH-stimulated antidiuresis, then the cytosolic or membrane-bound protein phosphatase (which can reverse the cyclic AMP-induced phosphorylation) serves as a modulator and/or terminator of the hormonal action.

7. Conclusion

In the foregoing studies we were able to demonstrate the existence of only a single protein phosphatase system and only a single protein kinase system that regulates multiple hormonal effects on the nephron. For each individual cell of renal tubule, there are probably only one or at most a few types of hormonal receptors, whereas the intracellular protein kinase(s) and phosphatase(s) probably have approximately the same distribution inside every cell. The question arises therefore as to what makes each cell have a selective response to a particular hormone. The final site of hormonal action may be a membrane-bound phosphoprotein--or set of such proteins--for each hormone. It would be interesting to compare membrane-bound phosphoproteins isolated from the different types of cells under the control of different peptide hormones in order to relate a particular molecule to a particular effector role.

In the case of parathyroid hormone, PTH stimulates phosphorylation of four cellular proteins (M_r = 150,000, 125,000, 100,000 and 50,000) in rabbit renal cortical tubules (250). Exposure of tubules to dibutyryl cyclic AMP had similar effects. In this crude preparation, however, it is impossible to determine the exact protein which may be involved in PTH action. Exposure of tubules to PTH did not affect protein dephosphorylation in a fashion analogous to the toad

bladder epithelial cells that were exposed to ADH. This difference may be due to the experimental method and procedure inasmuch as one expects PTH to raise the intracellular level of cyclic AMP and subsequently to increase the dephosphorylation of the regulatory subunit of type II protein kinase (which is the major protein kinase of renal tissue). Studies of the brush border isolated from rat kidney showed that a protein with a molecular weight of 35,000 was phosphorylated. These studies, however, failed to demonstrate cyclic AMP stimulation of the phosphorylation of this particular phosphoprotein. Kinne et al. (99) used free-flow electrophoresis to separate apical from basal-lateral plasma membrane. The basal-lateral membrane fraction contained PTH-sensitive adenylate cyclase activity. Protein kinase activity, as detected by phosphorylation of endogeneous membrane-bound substrate, was found predominantly in apical membranes but also to an appreciable degree in basal-lateral membranes. Basal protein kinase activity was higher in the apical membrane and was stimulated approximately two-fold by cyclic AMP; in the basal-lateral membranes, there was an approximately six-fold stimulation of protein kinase by cyclic AMP. Phosphorylation of membrane-bound protein was enhanced by the addition of a soluble cyclic AMP-dependent protein kinase isolated from the same tissue. In our unpublished work, we also found that there is membrane-bound protein phosphatase in the brush border. It appears that in renal tissue there are two sets of phosphorylation-dephosphorylation regulatory enzymes, one in the cytosol and the other in the plasma membrane. The former includes cyclic AMP-dependent protein kinase, protein phosphatase and phosphodiesterase; the latter,

cyclic AMP-dependent protein kinase and protein phosphatase. Although the cytosol fraction has approximately 65% of the total protein kinase and phosphatase activities, and the membrane fraction has only 5% of the total activities, it is very difficult to determine whether the cytosol or membrane-bound enzyme is involved in hormonal action in vivo. Since the kidney is involved in glycolysis, the cytosol enzyme may well also play a role in the regulation of glycogen metabolism. On the other hand, the membrane-bound enzyme may serve to activate the permeability change occurring immediately after the level of cyclic AMP is raised.

Up until the present time there has been no protein other than P-50 of which the state of phosphorylation is cyclic AMP-dependent and which is localized in the apical plasma membrane of the ADH target cell. There are several possible explanations of why no such cyclic-AMP generated phosphoprotein has yet been isolated:

a. The specific phosphoprotein may have been denatured during the process of isolation of plasma membrane, or the phosphoprotein may have become dissociated from the membrane during the purification process.

b. The cyclic AMP-dependent protein kinase may not have been able to gain access to the protein substrate in vitro, so that it was impossible to phosphorylate and to localize the phosphoprotein.

c. The phosphoprotein may have been rapidly dephosphorylated by membrane-bound phosphatase, since this enzyme is very active.

With improved techniques for studying the biochemistry of ADH action, one may be able to localize the specific membrane-bound phosphoprotein that is responsible for the ADH-induced change in membrane permeability.

Ueda _et al_. (139) have worked on the solubilization of a phosphoprotein and its associated cyclic AMP-dependent protein kinase and protein phosphatase from synaptic membrane fractions and on the basis of kinetic studies of endogeneous phosphorylation indicated that the enzyme system and phosphoprotein exist as a complex. If accumulating evidence continues to strengthen the hypothesis that the membrane-bound phosphorylation and dephosphorylation enzyme systems are involved in the ADH-induced change of permeability to water there should be an increased effort made to isolate the relevant phosphoprotein.

REFERENCES

1. Adolph, E.G., J.P. Barker and P.A. Hoy
Am. J. Physiol., 173:538-562 (1954)

2. Oliver, G. and Schafer, E.A.
J. Physiol., 18:277-279 (1895)

3. Dale, H.H.
J. Physiol., 34:163-206 (1906)

4. Ott, I. and Scott, J.C.
Proc. Soc. Exper. Biol. & Med., 8:48 (1910)

5. Paton, D.N. and Watson, A.
J. Physiol., 44:413-424 (1912)

6. Von den Velden, R.
Berl. klin. Wehnschr., 50:2083-2094 (1913)

7. Dubley, H.W.
J. Pharmacol., 14:295-312 (1919)

8. Dudley, H.W.
J. Pharmacol., 21:103-122 (1923)

9. Livermore, A.H. and du Vigneaud, V.
J. Biol. Chem., 180:365-373 (1949)

10. Pierce, J.G. and du Vigneaud, V.
J. Biol. Chem., 182:359-366 (1950)

11. Edman, P.
Acta Chem. Scandinav., 4:283 (1953)

12. du Vigneaud, V., Ressler, C. and Trippett, S.
J. Biol. Chem., 205:949-957 (1953)

13. Turner, R.A., Pierce, J.G. and du Vigneaud, V.
J. Biol. Chem., 191:21-28 (1951)

14. du Vigneaud, V., Lawler, H.C. and Popenoe, E.A.
J. Am. Chem. Soc., 75:4880-4881 (1953)

15. du Vigneaud, V.
Proc. Third Intl. Cong. Biochem., New York p. 49 (1955)

16. du Vigneaud, V.
Ann. N.Y. Acad. Soc., 88:537-548 (1960)

17. du Vigneaud, V., Gish, D.T., Katsoyannis, P.T., and Hess, G.P.
J. Am. Chem. Soc., 80:3355-3358 (1958)

18. Morgan, T. and Berliner, R.W.
Am. J. Physiol., 215:108-115 (1968)

19. Handler, J.S. and Orloff, J.
Handbook of Physiology, Sec., 8, p. 791-814
Edited by Orloff and R.W. Berliner, Am. Physiol. Soc.,
Washington, D.C. (1973)

20. Hays, R.M. and Levine, S.D.
Kidney Intl., 6:307-322 (1974)

21. Sawyer, W.H.
Pharmacol. Rev., 13:225-277 (1961)

22. Ussing, H.H., Kruhoffer, P., Thaysen, J.H. and Thorn, N.A.
The Alkali Metal Ions in Biology, p. 127
Berlin (1960), Springer-Verlag

23. Bentley, P.J.
Biol. Rev. 41:275-316 (1966)

24. Leaf, A.
J. Gen. Physiol. 43:175-189 (1960)

25. MacRobbie, E.A.C., and Ussing, H.H.

Acta Physiol. Scand., 53:348-365 (1961)

26. Leaf, A.

The Amer. J. Med., 42:745-756 (1967)

27. Hays, R.M. and Leaf, A.

J. Gen. Physiol., 45:905-919 (1962)

28. Ganoti, C.E., Grantham, J.J., Moses, H.L., Burg, M.B. and Orloff, J.

J. Cell. Biol., 36:355-367 (1968)

29. Morgan, T.F., Sakai, F. and Berliner, R,W,

Am. J. Physiol., 214:574-581 (1968)

30. Schafer, J.A. and Andreoli, T.E.

J. Clin. Invest., 51:1264-1278 (1972)

31. Capraro, V. and Bernini, G.

Nature 169:454 (1952)

32. Ussing, H.H. and Zerahn, K.

Acta. Physiol. Scand., 23:110-127 (1951)

33. Anderson, B. and Ussing, H.H.

Acta Physiol. Scand., 39:228-229 (1975)

34. Hay, R.M. and Leaf, A.

J. Gen. Physiol., 45:933-948 (1962)

35. Leaf, A. and Hays, R.M.

J. Gen. Physiol., 45:921-932 (1962)

36. Lichtenstein, N.S. and Leaf, A.

Ann. N.Y. Acad. Sci., 137:556-565 (1966)

37. Lichtenstein, N.S. and Leaf, A.

J. Clin. Res., 23:368 A (1965)

38. Athar, S. and Robertson

Clin. Res., 22:335A (1974)

39. Pitts, R.F.

Physiology of the Kidney and Body Fluid, Year Book Medical Publisher, Chicago (1973)

40. Strewler, G.J. and Orloff, J.

J. Advance Cyclic Nucleotide Res., Vol. 8, 311-381, Raven Press, N.Y. (1977)

41. Ullrich, K.J.

Arch. Ges. Physiol., 261:62 (1955)

42. Grantham, J.J.

Science, 168:1093-1095 (1970)

43. Grantham, J.J. and Burg, M.E.

Am. J. Physiol., 211:255-259 (1966)

44. Burg, M., Helman, S.I., Grantham, J. and Orloff, J.

In: Urea and Kidney, Ed., B. Schmidt-Nielsen, D.W., S. Kerr, p. 193-198, Amsterdam Excerpta Medica, 495 P.P (1970)

45. Jasenike, J.R.

J. Clin. Invest. 30:144-151 (1961)

46. Fromter, E. and Diamond, J.

Nature New Biol., 235:9-12 (1972)

47. Andreoli, T.E. and Schafer, J.A.

Ann. Rev. Physiol., 39:451-500 (1976)

48. Leaf, A., Anderson, J. and Page, L.P.

J. Gen. Physiol., 41:657-658 (1958)

49. Civan, M.M. and DiBona, D.R.
J. Memb. Biol., 19:195-220 (1974)

50. Wright, D.W. and Snart, R.S.
Life Sci., 10:301-308 (1971)

51. Schafer, J.A. and Andreoli, T.E.
J. Clin. Invest., 51:1278-1286 (1972)

52. Rocha, A. and S. Kokko
Kidney Intl., 6:379-387 (1974)

53. Frindt, G. and Burg, M.B.
Kidney Intl., 1:224-231 (1972)

54. Helman, S.I., Grantham, J.J. and Burg, M.B.
Am. J. Physiol., 220:1825-1832 (1971)

55. Levine, S.D., Franki, N., and Hays, R.M.
J. Clin. Invest., 52:1425-1442 (1973)

56. Levine, S.D., Kaplan, M.D., Franki, N., Meyers, J.. and Hays, R.M.
Bull. Desert. Isl. Dio. Lab., 13:70-71 (1973)

57. Schuchter, S.H., Franki, N. and Hays, R.M.
J. Memb. Biol., 14:177-191 (1973)

58. Peterson, M.J. and Edelman, I.S.
J. Clin. Invest., 43:583-594 (1964)

59. Bentley, P.J.
Gen. Comp. Endocrinol., 13:39-44 (1969)

60. Maetz, J.
Symp. Zool. Soc., London, 9:107-140 (1963)

61. Lipson, L.C. and Sharp, G.W.G.
Am. J. Physiol., 220:1046-1052 (1971)

62. Mamelak, M., Weissbluth, M. and Maffy, R.H.
Biochem. Pharmacol., 19:2303-2315 (1970)

63. Taylor, A.
Fed. Proc., 34:285 (1975) (Abstr.)

64. DeSousa, R.C., Gorsso, A. and Rufener, C.
Experientia, 15:175-177 (1974)

65. Taylor, A., Mamelak, M., Reaven, E. and Maffly, R.
Science, 181:347-350 (1973)

66. Marumo, F.
Pfluegers Arch., 299:149-157 (1968)

67. Levine, S.D., Levine, R.D., Osthington, R.E. and Hays, R.M.
Clin. Res., 23:432A (1975)

68. Pauling, L.
Science, 134:15-21 (1961)

69. Parrish, R.G. and Kurland, R.J.
Biophys. Soc. Abstr., 15:303 (1975)

70. Schafer, J.A., Troutman, S.L. and Andreoli, T.E.
J. Gen. Physiol., 64:229-240 (1974)

71. Andreoli, T.E.
Kidney Int. 4:337-345 (1973)

72. Pietras, R.J. and Wright, E.M.
Nature, London 247:222-224 (1974)

73. Bentley, P.J.
J. Physiol., London, 195:317-330 (1968)

74. Ohno, T., Goodman, D.B.P., and Rasmussen, H.
J. Memb. Biol., 18:295-304 (1974)

75. Bentley, P.J.
J. Pharmacol. Exp. Ther., 189:563-569 (1974)

76. Bentley, P.J. and Wasserman, A.
Biochem. Biophys. Acta, 266:285-292 (1972)

77. Koefoed-Johnsen, V. and Ussing, H.H.
Acta. Physiol. Scand., 28:60-76 (1958)

78. Frazier, H.S., Dempsey, E.F. and Leaf, A.
J. Gen. Physiol., 45:529-533 (1962)

79. Crabbe, J. and DeWeer, P.
J. Physiol., London, 180:560-568 (1965)

80. Frazier, H.S. and Leaf, A.
Medicine, 43:281-289 (1964)

81. Curran, P.F., Herrera, F.C. and Flanigan, W.J.
J. Gen. Physiol., 46:1011-1027 (1963)

82. Civan, M.M. and Frazier, H.S.
J. Gen. Physiol., 51:589-605 (1968)

83. Rawlins, F., Mateu, L., Fragachan, F. and Whittenburg, G.
Pflueger Arch., 316:64-80 (1970)

84. Kidder, G.W., Cereijido, M. and Curran, P.F.
Am. J. Physiol., 207:935-940 (1964)

85. Hays, R.M. and Franki, N.
J. Memb. Biol., 2:263-276 (1970)

86. Orloff, J. and Handler, J.S.
Biochem. Biophys. Res. Commun., 5:63-66 (1961)

87. Orloff, J. and Handler, J.S.
J. Clin. Invest., 41:702-709 (1962)

88. Handler, J.S. and Orloff, J.
Am. J. Physiol., 205:298-302 (1963)

89. Bockaert, J., Roy, C., Rajerison, R. and Jard, S.
J. Biol. Chem., 248:5922-5931 (1973)

90. Dousa, T., Hechter, O., Walter, R., Schwartz, I.L.
Science, 167:1134-1135 (1970)

91. Cuatrecasas, P.
Ann. Rev. Biochem., 43:169-214 (1974)

92. Berde, B. (Ed.)
Neurohypophyseal Hormones and Similar Polypeptides
Handbuch der Pharmakologie, Vol. 23 (1968)

93. Fergusion, M.G. and Price, R.H.
Adv. Cyclic Nucleotide Res., 1:113-119 (1972)

94. Dousa, T.P., Sands, H., and Hechter, O.
Endocrinology, 91:757-763 (1972)

95. Handler, J.S., Butcher, R.W., Sutherland, E.W. and Orloff, J.
J. Biol. Chem., 240:4524-4526 (1965)

96. Campbell, B.J., Woodward, G. Borberg, V.
J. Biol. Chem., 247:6167-6175 (1972)

97. Dousa, T.P., Walter, R., Schwartz, I.L., Sands, H. and Hechter, O.
Adv. cyclic Nucleotide Res., 1:121-135 (1972)

98. Handler, J.S. and Orloff, J.
Ann. N.Y. Acad. Sci. 185:345-350

99. Schwartz, I.L., Shaltz, L.J., Kinne-Saffran, E., and Kinne, R.
Proc. Natl. Acad. Sci. USA, 71:2595-2599 (1974)

100. Rajerison, R., Marchetti, J., Roy, C., Bochaert, J., and Jard, S.
J. Biol. Chem., 249:6390-6400 (1974)

101. Rall, T.W., Sutherland, E.W. and Berthel, J.
J. Biol. Chem., 224:463-475 (1957)

102. Walsh, D.A., Perkins, J.P. and Kreb, E.G.
J. Biol. Chem., 243:3763-3765 (1968)

103. Robinson, G.A., Butcher, R.W. and Sutherland, E.W.
Cyclic AMP, Academic Press, N.Y. (1971)

104. Langan, T.A.
Adv. Cyclic Nucleotide Res., 3:99-153 (1973)

105. Kuo, J.F. and Greengard, P.
Proc. Natl. Acad. Sci., U.S.A., 64:1349-1355 (1969)

106. Krebs, E.G.
Current Topics of Cell Regul., 5:99-133 (1972)

107. Kreb, E.G.
Endocrinology. Proc. 4th Intl. Cong., p. 17-29, Excerpta Medica, Amsterdam (1973)

108. Nimmo, H.G. and Cohen, P.
Advance in Cyclic Nucleotide Res., Vol. 8, p. 145-265 Raven Press, N.Y. (1977)

109. Rubin, C.S. and Rosen, O.M.
Ann. Rev. Biochem., 44:831-887 (1975)

110. Lehninger, A.L.
Biochemistry, Worth Publisher, Inc., (1975)

111. Villar-Palasi, C. and Larner, J.
Arch. Biochem. Biophys., 94:436-442 (1961)

112. Rosell-Perez, M., Villar-Palasi, C. and Larner, J.
Biochim. Biophys. Acta, 242:81-88 (1971)

113. Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A. and Krebs, E.G.
J. Biol. Chem., 245:6317-6328 (1970)

114. Stadtman, E.R. and Chock, P.B.
Proc. Natl. Acad. Sci., U.S.A., 74:2761-2765 (1977)

115. Chock, P.B. and Stadman, E.R.
Proc. Natl. Acad. Sci., U.S.A. 74:2761-2765 (1977)

116. Roach, P.J.
Trends Biochem. Sci., 2:87-90 (1977)

117. Chen, C.-C., Bruegger, B.B., Kern, C.W., Lin, Y.C., Halpern, R.M. and Smith, R.A.
Biochemistry, 16:4852-4855 (1977)

118. Smith, L.S., Kern, C.W., Halpern, R.M. and Smith, R.A.
Biochem. Biophys. Res. Commun., 71:456-465 (1976)

119. Smith, D.L., Bruegger, B.B., Halpern, R.M. and Smith, R.A.
Nature, London, 246:103-104 (1973)

120. Walsh, D.A., Ashby, C.D., Gonzalex, C., Calkins, D., Fisher, E.H. and Krebs, E.G.
J. Biol. Chem., 246:1977-1985 (1971)

121. Walsh, D.A., Ashby, C.D. and Walsh, D.A.
Methods Enzymol., 38C:290-299 (1974)

122. Kuo, J.F., Krueger, B.K., Sanes, J.R. and Greengard, P.
Biochim. Biophys. Acta, 212:79-91 (1970)

123. Beavo, J.A., Bechtel, P.J. and Krebs, E.G.
Methods Enzymol., 38C:299-308 (1974)

124. Rubin, C.S., Erlichman, J. and Rosen, O.M.
J. Biol. Chem., 247:36-44 (1972)

125. Kuo, J.F.
Proc. Natl. Acad. Sci., U.S.A. 71:4037-4041 (1974)

126. Miyamoto, E., Petzold, G.L., Kuo, J.F. and Greengard, P.
J. Biol. Chem., 248:179-189 (1973)

127. Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyama, K., Yamamura, H. and Nishizuka, Y.
J. Biol. Chem., 251:1481-1487 (1976)

128. Langan, T.A.
Proc. Nat. Acad. Sci., U.S.A. 64:1276-1283 (1969)

129. Rosen, O.M., Erlichman, J. and Rubin, C.S.
Adv. Cyclic Nucleotide Res., 5:253-263 (1975)

130. Beavo, J.A., Bechtel, P.J. and Krebs, E.G.
Adv. Cyclic Nucleotide Res., 5:241-251 (1975)

131. Rubin, C.S., Erlichman, J. and Rosen, O.M.
Method in Enzymol., 38C:308-315 (1974)

132. Lincoln, T.M., Dills, W.L. and Corbin, J.P.
J. Biol. Chem. 252:4296-4257 (1977)

133. Corbin, J.D., Keely, S.L. and Park, C.R.
J. Biol. Chem., 250:218-225 (1975)

134. Corbin, J.D. and Keely, S.L.
J. Biol. Chem. 252:910-918 (1977)

135. Hoffman, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G.
J. Biol. Chem., 250:7785-7801 (1975)

136. Erlichman, F., Rosenfeld, R. and Rosen, O.M.
J. Biol. Chem., 249:5000-5003 (1974)

137. Rosen, O.M. and Erlichman, J.
J. Biol. Chem., 250:7788-7794 (1975)

138. Chou, C.K., Alfano, J. and Rosen, O.M.
J. Biol. Chem., 252:2855-2859 (1977)

139. Uno, I., Ueda, T., and Greengard, P.
J. Biol. Chem., 252:5164-5174 (1977)

140. Holzer, H. and Duntze, W.
Ann. Rev. Biochem., 40:345-374 (1971)

141. Segel, H.L.
Science, 180:25-32 (1973)

142. Shalteil, S., (Ed.)
Metabolic Interconversions of Enzyme, 1975
Springer-Verlag, N.Y. (1975)

143. Fisher, E.H., Krebs, E.G., Neurath, H. and Stadtman, E.R. (Eds.)
Metabolic Interconversions of Enzyme, 1973
Springer-Verlag, N.Y. (1974)

144. Hujing, F. and Lee, E.Y.C. (Eds.)
Protein Phosphorylation in Control Mechanism
Academic Press, N.Y. (1973)

145. Jard, A. and Bastide, F.
Biochem. Biophys. Res. Commun., 39:559-566 (1970)

146. Kirchberger, M.A., Schwartz, I.L. and Walter, R.
Proc. Soc. Exp. Biol. Med., 140:657-660 (1972)

147. DeLorenzo, R.J., Walton, K.G., Curran, P.F. and Greengard, P.
Proc. Natl. Acad. Sci., U.S.A., 70:880-884 (1973)

148. DeLorenzo, R.J. and Greengard, P.
Proc. Natl. Acad. Sci., U.S.A., 70:1831-1835 (1973)

149. Walton, K.G., DeLorenzo, R.J., Curran, P.F. and Greengard, P.
J. Gen. Physiol., 65:153-177 (1975)

150. Ferguson, D.R. and Twite, B.R.
J. Endocrinol., 61:501-507 (1974)

151. Malkinson, A.M., Kraeger, B.K., Rudolph, S.A., Cashellie, J.E., Haley, B.E. and Greengard, P.
Metabolism, 24:331-341 (1975)

152. Rubin, C.S.
J. Biol. Chem., 250:9044-9052 (1975)

153. Liu, A.Y-C. and Greengard, P.
Proc. Natl. Acad. Sci., U.S.A., 71:3869-3873 (1974)

154. Liu, A.Y-C. and Greengard, P.
Proc. Natl. Acad. Sci., U.S.A., 73:568-572 (1976)

155. Handler, J.S., Preston, A.S. and Orloff, J.
J. Clin. Invest., 48:823-833 (1969)

156. Manganiello, V. and Vaughan, M.
J. Clin. Invest., 51:2763-2768 (1972)

157. Taylor, A., Maffly, R., Wilson, L. and Reaven, E.
Ann. N.Y. Acad. Sci., 253:723-737 (1975)

158. Dousa, T.P., Barnes, L.D. and Jin, K.K.
Neurohypophysis. Int. Conf., Key Biscayne, Fla., p. 220-235 (1976)

159. Stephens, R.E. and Edds, K.T.
Physiol. Rev., 56:709-777 (1970)

160. Dousa, T.P. and Barnes, L.D.
Proc. Soc. Exp. Biol. Med., 31:824 (1972)

161. Hall, D., Taylor, A. and Maffly, R.
Kidney Int., 6:49A (1974)

162. Barnes, L.D., Engel, A.G. and Dousa, T.P.
Biochim. Biophys. Acta, 405:422-433 (1975)

163. Barnes, L.D., Hui, Y.S.F. and Dousa, T.P.
Physiologist, 18:131 (1975)

164. Barnes, L.D., Hui, Y.S.F. and Dousa, T.P.
Clin. Res., 23:504A (1975)

165. Dousa, T.P. and Barnes, L.D.
Am. J. Physiol., 231(1):F50-F57 (1977)

166. Masur, S.K., Holtzman, E. and Walter, R.
J. Cell Biol., 52:211-219 (1972)

167. DiBona, D.R., Civan, M.M. and Leaf, A.
J. Memb. Biol., 1:79-91 (1969)

168. Danon, D., Strum, J.M. and Edelman, I.S.
J. Memb. Biol., 16:279-294 (1974)

169. Pietras, R.J., Naujukaitis, P.J. and Szego, C.M.
Molec. & Cell. Endocrinol., 4:89-106 (1976)

170. DiBona, D.R., Civan, M.M. and Leaf, A.
J. Cell Biol., 40:1-7 (1969)

171. Sharp, G.W. and Leaf, A.
Physiol. Rev., 46:593-633 (1966)

172. Handler, J.S. and Preston, A.S.
J. Memb. Biol., 26:43-50 (1976)

173. Scott, W.N., Sapirstein, V.S. and Yoder, M.J.
Science, 184:797-799 (1974)

174. Goodman, D.B.P., Bloom, F.E., Battenberg, E.R., Rasmussen, H., and Davis, W.L.
Science, 188:1023-1025 (1975)

175. Zusman, R.M., Keiser, H.R. and Handler, J.S.
J. Clin. Invest., 60:1339-1347 (1977)

176. Omachi, R.S., Robbre, J.E., Handler, J.S. and Orloff, J.
Am. J. Physiol., 226:1152-1157 (1974)

177. Lipson, L.S., Hynie, S. and Sharp, G.W.G.
Ann. N.Y. Acad. Sci., U.S.A., 180:261-277 (1971)

178. Peachey, L.D. and Rasmussen, H.
J. Biophys. Biochem. Cytol., 10:529 (1961)

179. Margacet, A., Ekblad, E.B., Strum, J.M. and Edelman, I.S.
J. Memb. Biol., 26:301-317 (1976)

180. Pisam, M., Ripoche, P. and Rambourg, A.
C.R. Acad. Sci., (Paris) Ser. D., 271:105 (1970)

181. Pisam, M. and Ripoche, P.
J. Microsc. (Paris), 17:261-282 (1973)

182. Pisam, M. and Ripoche, P.
J. Cell Biol., 71:907-920 (1976)

183. Chevalier, J., Bourguet, J. and Hugon, J.S.
Cell Tiss. Res., 152:129-140 (1976)

184. Wade, J.B., DiScala, V.A. and Karnovsky, M.J.
J. Memb. Biol., 22:385-402 (1975)

185. Kachadorin, W.A., Wade, J.B., Niterwyk, C. and DiScala, V.A.
J. Memb. Biol., 30:381-401 (1977)

186. Kachadorin, W.A., Casey, C. and DiScala, V.A.
Am. J. Physiol., 234(6), F461-465 (1975)

187. Pietras, R.J. and Wright, E.M.
Nature, 247:222-224 (1974)

188. Pietras, R.J. and Wright, E.M.
J. Memb. Biol., 22:107-123 (1975)

189. Al-Zahid, G., Schafer, J.A. and Andreoli, T.E.
The Physiologist, 18:120 (1975)

190. Pietras, R.J., Steeler, B.J. and Sego, C.M.
Nature, 257:493-495 (1975)

191. Solyom, A. and Trans, E.G.
Enzyme, 13:329-372 (1972)

192. Fiske, C.H. and Subbarow, Y.
J. Biol. Chem., 234:466-468 (1925)

193. Hushscher, G. and West, G.R.
Nature, 205:779-780 (1965)

194. Gibbs, G.E. and Reiner, K.
Proc. Soc. Exp. Biol. Med., 119:470-478 (1965)

195. Bar, H-P. and Hechter, O.
Anal. Biochem., 29:476-489 (1969)

196. Lowry, O.H., Rosebraugh, N.J., Farr, A.L. and Randall, R.J.
J. Biol. Chem., 193:265-275 (1951)

197. Watson, M.L.
J. Biophys. Biochem. Cytol., 4:474-478 (1958)

198. Venable, J.H. and Coggeshall, R.A.
J. Cell. Biol., 25:407-408 (1965)

199. Li, H.C. and Felmly, D.A.
Analytical Biochem., 52:300-304 (1973)

200. Gilman, A.G.
Proc. Natl. Acad. Sci., U.S.A., 67:305-308 (1970)

201. Reimann, E.M., Walsh, D.A. and Krebs, E.G.
J. Biol. Chem., 250:7795-7801 (1971)

202. Miyamoto, E., Kuo, J.F. and Greengard, P.
J. Biol. Chem., 244:6395-6402 (1969)

203. Meisler, M.H. and Langan, T.A.
J. Biol. Chem., 244:4961-4968 (1969)

204. Torres, H. and Chelala, C.A.
Biochim. Biophys. Acta. 198:475-503 (1970)

205. Hisao, K.J., Chan, W.W.S. and Li, H.C.
Biochim. Biophys. Acta, :337-347 (1977)

206. David, B.J. and Ornstein, R.
Ann. N.Y. Acad. Sci., 121:404-407 (1964)

207. Laemmli, U.K.
Nature (London), 227:680-685 (1970)

208. Fairbanks, G., Steck, T.L. and Wallach, D.F.H.
Biochemistry, 10:2606-2617 (1971)

209. Reid, M.S. and Bielski, R.L.
Anal. Biochem., 22:374-381 (1968)

210. Pepierre, J.W. and Karnovsky, M.L.
J. Cell. Biol., 56:275-303 (1973)

211. Fitzpatrick, D.F., Davenport, R.D., Forte, L. and Landon, E.J.
J. Biol. Chem., 244:3561-3569 (1969)

212. Martin, R.G. and Ames, B.N.
J. Biol. Chem. 236:1372-1379 (1961)

213. Sutherland, E.W.
Harvey Lec., 57:17-33 (1961-1962)

214. Chase, L.R. and Aurbach, G.D.
Science, 159:545-547 (1968)

215. Marcus, R. and Aurbach, G.D.
Endocrinol., 85:801-810 (1969)

216. Melson, G.L., Chase, L.R. and Aurbach, G.D.
Endocrinol., 86:511-518 (1970)

217. Neer, E.J.
J. Biol. Chem., 248:4775-4781 (1973)

218. Chabardes, D., Imbert-Tebonl, M., Montegut, M., Clique, A.
and Morel, F.
Pflugers Arch., 361:9-15 (1975)

219. Chabardes, D., Imbert, M. and Morel, F.
Intl. Workshop on Phosphate, Paris (1975)

220. Chabardes, D., Imbert, M., Clique, A., Montegut, M. and Morel, F.
Pflugers Arch., 354:229-239 (1975)

221. Imbert, M., Chabardes, D., Montegut, M., Clique, A. and Morel, F.
Pflugers Arch., 357:173-186 (1975)

222. Imbert, M., Chabardes, D., Montegut, M., Clique, A. and Morel, F.
Pflugers Arch., 354:213-228 (1975)

223. Morel, F.. Chabardes, D. and Imbert, M.
Curr. Prob. Clin. Biochem., 6:214-222 (1976)

224. Berl, T., Harbottle, J.A., and Schrier, R.W.
Kidney Intnl., 6:247-253 (1974)

225. Lee, P.
Brit, J. Pharmacol., 34:429-444 (1968)

226. Ueland, P.M. and Døskeland, S.O.
J. Biol. Chem., 252:677-686 (1977)

227. Yuh, K-C.M. and Tao, M.
Biochemistry, 13:5520-5526 (1974)

228. Hsiao, K.J., Chan, W.W.S. and Li, H.J.
Biochim. Biophys. Acta, 483:337-341 (1977)

229. Killilea, S.D., Brandt, H., Lee, E.Y.C. and Whelan, W.J.
J. Biol. Chem., 251:2363-2368 (1976)

230. Lee, E.Y.C., Brandt, H., Capulong, Z.L. and Killilea, S.D.
Adv. Enzyme. Regul., 14:467-470 (1975)

231. Gratecos, D., Detwiler, T.C., Hurd, S. and Fisher, E.H.
Biochemistry, 16:4812-4817 (1977)

232. Killilea, S.D., Brandt, H. and Lee, E.Y.C.
Trends Biochem. Sci., 1:30-33 (1976)

233. Rangel-Aldao, R. and Rosen, O.M.
J. Biol. Chem., 251:7526-7529 (1976)

234. Brant, H., Lee, E.Y.C., and Killilea, S.D.
Biochem. Biophys. Res. Commun., 63:950-956 (1975)

235. Brandt, H., Killilea, S.D. and Lee, E.Y.C.
Biochem. Biophys. Res. Commun., 61:548-554 (1974)

236. Kato, K., Kobayashi, M. and Sato, S.
J. Biochem. (Tokyo), 77:811-815 (1975)

237. Merlevede, W., Goris, J. and De Brandt, C.
Eur. J. Biol., 11:499-502 (1969)

238. Li, H.C.
FEBS Letter, 55:134-137 (1975)

239. Paietta, E. and Sands, H.
Biochim. Biophys. Acta, 521:121-132 (1978)

240. Miyamoto, E. and Kakiuchi, S.
Biochim. Biophys. Acta., 384:458-465 (1975)

241. Nakai, C. and Thomas, J.A.
J. Biol. Chem., 249:6459-6467 (1974)

242. Traugh, J.A. and Sharp, S.B.
J. Biol. Chem., 252:3738-3744 (1977)

243. Huang, F.L. and Glinsmann, W.H.
Proc. Natl. Acad. Sci., U.S.A., 72:3004-3008 (1975)

244. Huang, F.L. and Glinsmann, W.H.
Eur. J. Biochem., 70:419-425 (1976)

245. Kato, K. and Sato, S.
Biochim. Biophys. Acta., 358:299-307 (1974)

246. Grave, P.H., Fisher, E.H. and Krebs, E.G.
J. Biol. Chem., 235:805-809 (1960)

247. Kandelwal, R.L., Vandenheeds, J.R. and Krebs, E.G.
J. Biol. Chem., 251:4850-4858 (1976)

248. Kikuchi, K., Tamura, S., Hiraga, A. and Tsuiki, S.
Biochem. Biophys. Res. Commun., 75:29-39 (1977)

249. Antoniw, J.F. and Cohen, P.
Eur. J. Biochem., 68:45-54 (1976)

250. Ausillo, D., Handler, J.S. and Orloff, J.
Biochim. Biophys. Acta, 451:372-381 (1976)

251. Siegel, L.M. and Monty, K.J.
Biochim. Biophys. Acta., 112:346-362 (1966)